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Perspectives for Forensic Intelligence in Anti-Doping and the emergence of smokeless tobacco consumption in sport

François Marclay

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Le Président du Jury

Professeur Christophe Champod

Lausanne, le 11 juin 2014

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FACULTÉ DE BIOLOGIE ET DE MÉDECINE
Professeur Martial Saugy
Professeur Patrice Mangin

Perspectives for Forensic Intelligence in Anti-Doping and the emergence of smokeless tobacco consumption in sport

THÈSE

présentée à l'Institut de Police Scientifique, Ecole des Sciences Criminelles, Faculté de Droit, des Sciences Criminelles et d'Administration Publique de l'Université de Lausanne pour obtenir le grade de Docteur ès sciences, mention sciences forensiques

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"We are all apprentices in a craft where no one ever becomes a master."
- Ernest Hemingway

List of abbreviations

Organizations

AAPS	Association of Pharmaceutical Scientists
ADCH	Antidoping Switzerland
BALCO	Bay Area Laboratory Co-Operative
CAS	Court of Arbitration for Sport
CHUV	Centre Hospitalier Universitaire Vaudois
CRC	Centre de Recherche Clinique - CHUV
EPFL	Ecole Polytechnique Fédérale de Lausanne
ESC	Ecole des Sciences Criminelles – UNIL
EUROPOL	European Police Office
FDA	Food and Drug Administration
FIFA	Fédération Internationale de Football Association
FISA	International Rowing Federation
HS	European Healthy Stadia Network
IAAF	International Association of Athletics Federations
IF	International Sports Federation
IIHF	International Ice Hockey Federation
INTERPOL	International Criminal Police Organization
IOC	International Olympic Committee
ISSUL	Institute of Sport Sciences – UNIL
IST	Institut de Santé au Travail - CHUV
IUMSP	Institut Universitaire de Médecine Sociale et Préventive - CHUV
LAD	Swiss Laboratory for Doping Analyses - CHUV
MiLB	American Minor League Baseball
MLB	American Major League Baseball
NADO	National Anti-Doping Organization
NCAA	National College Athletic Association
NCI	National Cancer Institute
PBATS	Professional Baseball Athletic Trainers Society
PMU	Polyclinique Médicale Universitaire - CHUV
RADO	Regional Anti-Doping Organization
ROC	Russian Organized Crime

UCI	Union Cycliste Internationale
UCLA	University of California Los Angeles
UEFA	Union of European Football Associations
UNIL	University of Lausanne
USADA	United-States Anti-Doping Agency
WAADS	World Association of Anti-Doping Scientists
WADA	World Anti-Doping Agency
WHO	World Health Organization

Anti-Doping related terms

AAF	Adverse Analytical Finding
AAS	Anabolic Androgenic Steroids
ABP	Athlete Biological Passport
APF	Adverse Passport Finding
hCG	human Chorionic Gonadotropin
HGH	Human Growth Hormone
IC	In-Competition
ISL	International Standard for Laboratories
MRPL	Minimum Required Performance Levels
OOC	Out-of-Competition
rEPO	Human Recombinant Erythropoietin
SERMs	Selective Estrogen Receptor Modulators
THC	Tetrahydrocannabinol
THG	Tetrahydrogestrinone
TUEs	Therapeutic Use Exemptions

Tobacco-related terms

CYP	Cytochrome P-450
e-cigarette	Electronic Cigarette
ETS	Environmental Tobacco Exposure
FMO-3	Flavin Monooxygenase-3
MoNIC	Moniteur de NICotine
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

PAH	Polycyclic Aromatic Hydrocarbons
ST	Smokeless Tobacco
TSNA	Tobacco-Specific Nitrosamines
UGT	Uridine 5′-diphosphoglucuronosyltransferase

Analytical chemistry

ACN	Acetonitrile
API	Atmospheric Pressure Ionization
DS	Dilute-and-Shoot
ESI	Electrospray Ionization
F5	Pentafluorophenylpropyl
HILIC	Hydrophilic Interaction Chromatography
IS	Internal Standard
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
MCX	Mixed-mode Cation Exchange Solid-Phase Extraction
ME	Matrix Effect
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
PE	Process Efficiency
QC	Quality Control
RE	Extraction Efficiency
RSD	Relative Standard Deviation
SPE	Solid-Phase Extraction
SRM	Single Reaction Monitoring
t_R	Time Retention
UHPLC-MS/MS	Ultra-High Pressure Liquid Chromatography-triple Quadrupole Mass Spectrometry
ULOQ	Upper Limit of Quantification
Uneg	Negative Urine

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CHAPTER I | INTRODUCTION

I.1. Anti-Doping in Sport

I.1.1. Genesis of the fight against doping

In 1928, the International Amateur Athletics Federation (now the International Association of Athletics Federations) became the first International Federation to ban the use of stimulants in competition (1). The real starting point for anti-doping programs occurred only later on, when the International Olympic Committee (IOC) formed a Medical Commission in 1967, which supervised the introduction of anti-doping tests for stimulants the following year during the 1968 Summer Olympic Games in Mexico City. The same year saw the adoption of the first Anti-Doping Convention of the Council of Europe.

Two decades later, in 1986, the International Olympic Charter against Doping in Sport was inaugurated by the IOC, quickly followed by a revision of the Anti-Doping Convention and its acceptance by the Council of Europe in 1989 (1). The fight against doping found a cornerstone in the second version of this document as it initiated a crucial dialogue between governments and sport authorities for the international harmonization of anti-doping activities.

With an initial impulse from the IOC and as a response to the doping scandals at the Tour de France throughout the 90s and in particular the 1998 Festina affair, the World Anti-Doping Agency (WADA) was founded in December 1999 (1). In 2004, the first edition of the World Anti-Doping Code (the Code) received international acceptance as the regulation to rule anti-doping world-wide, replacing the IOC guidelines previously established (2). The second and latest edition of the Code has been released in 2009 (3). Since 2004, the WADA List Committee constituted of a panel of experts has been in charge of maintaining and proposing an annual update of the List of Prohibited Substances and Methods (the List) (4). In parallel, a Monitoring Program was also launched to monitor the use of permitted substances with presumably doping potential through analytical monitoring and to assess the need for regulation of these substances (5).

Subsequently, National and Regional Anti-Doping Organizations (NADOs and RADOs) were created to coordinate the collaboration between International Federations and governmental bodies in their joint efforts against doping in sport. Similarly, the World Association of Anti-Doping Scientists (WAADS) was established to promote the exchange of scientific knowledge and expertise between researchers of WADA Accredited Laboratories as well as for expressing problems and finding solutions in a community driven fashion. In 1983, the Court of Arbitration in Sport (CAS) was constituted in Lausanne (Switzerland) as the supreme authority in sport to prosecute disputed cases and to ensure a complete harmonization of the sanctions among the International Federations at an international level (1).

I.1.2. Anti-Doping legislation

A. The World Anti-Doping Code

The spirit of sport, considered as the essence of Olympism, is the celebration of the human spirit, body and mind and its intrinsic values such as ethic, fair play, honesty, respect, dedication and commitment, health or excellence in performance. Doping is fundamentally contrary to this ideal and is defined as the occurrence of one or more of the eight anti-doping rule violations set in the 2nd edition of the Code revised in 2009 (3):

1. Presence of a prohibited substance or its metabolites or markers in an athlete's biological sample;
2. Use or attempted use by an athlete of a prohibited substance or method;
3. Refusing or failing without compelling justification to submit to sample collection after notification, or otherwise evading sample collection;
4. Violation of applicable requirements regarding athlete availability for out-of-competition testing, including failure to file required whereabouts information and missed tests;
5. Tampering or attempted tampering with any part of doping control;
6. Possession of prohibited substances or methods;
7. Trafficking or attempted trafficking of any prohibited substance or method;
8. Administration or attempted administration to any athlete in-competition of any prohibited method or substance, or to any athlete out-of-competition of any substance or method prohibited out-of-competition, or any type of complicity involving an anti-doping rule violation or any attempted anti-doping rule violation.

Thus, the world anti-doping program, established by the Code, seeks to protect the athletes' fundamental right to participate in a doping-free sport and thus promotes health, fairness and equality for athletes worldwide, but also to ensure harmonized, coordinated and effective anti-doping programs at the national and international level with regard to detection, deterrence and prevention of doping. Athletes with a sport license have to accept these fundamental rules as a condition of participation and are bound by these rules.

Noteworthy, the 3rd revision of the Code due to come in effect in 2015 will contain additional emphasis on the fight against the complicity to doping and an increased length of the two-years ban into a four-years ban.

B. The International Standards

The Code works together with five International Standards harmonizing various technical processes related to the practice of anti-doping. These documents are the List of Prohibited

Substances and Methods (the List), the International Standard for Testing, the International Standard for Laboratories (ISL), the Therapeutic Use Exemptions (TUEs) and the International Standard for the Protection of Privacy and Personal Information.

As a laboratory, the List and the ISL are two documents of outmost importance (4, 6). An annual review of the List is conducted by a committee of international experts to update the document with additional substances or, conversely, to remove substances (4). The revised document comes in force on the 1st of January each year. In order whether to include a substance or method on the List, at least two of the following three criteria must be fulfilled (3):

1. Medical or other scientific evidence, pharmacological effect or experience that the substance or method, alone or in combination with other substances or methods, has the potential to enhance or enhances sport performance;
2. Medical or other scientific evidence, pharmacological effect or experience that the use of the substance or method represents an actual or potential health risk to the athlete;
3. WADA's determination that the use of the substance or method violates the spirit of sport described in the introduction to the Code.

The List is divided into 10 classes of substances (anabolic agents, peptide hormones, growth factors, stimulants, diuretics, etc.), 3 classes of methods (blood manipulation, gene doping, etc.), and 2 classes of substances prohibited in specific sports only (alcohol and β-blockers) (4). In 2014, close to 700 substances appear on the List and may be prohibited either in-competition (IC) or at all times.

The purpose of the ISL is to ensure the production of valid test results and evidentiary data and to achieve uniform and harmonized results and reporting from all laboratories (6). Requirements for obtaining and maintaining WADA accreditation are also included, as well as operating standards for laboratory performance and a description of the accreditation process. As of March 2014, 32 laboratories around the world are WADA Accredited, among which the Swiss Laboratory for Doping Analyses (LAD) of Lausanne, Switzerland.

In addition, specific technical recommendations are described in Technical Documents on various topics such as the Decision Limits for the Confirmatory Quantification of Threshold Substances, the Minimum Required Performance Levels (MRPL) or the Identification Criteria for Qualitative Assays. The current ISL is in force since the 1st of January 2012 and is mandatory for all signatories of the Code.

I.1.3. Today's approach and challenges of Anti-Doping

In order to meet the technological requirements linked to the continuous evolution of the List, research in anti-doping is mostly focused on the analysis of products in biological fluids. In

consequence, the field piles up with analytical tools and methodologies, rather than offering new perspectives and a fundamental look into the phenomenon (original paper in Appendix V.2.1 and V.2.2.). Indeed, WADA Accredited Laboratories have to spend most of their resources on developing more sensitive and selective methods with the latest and most sophisticated technology. Alternatively, the community has paid only peripheral attention to understanding the phenomenon as a whole using strong scientific data and alternative sources of information. Technology serves as an essential tool to today's approach which is almost strictly centered on the anti-doping disciplinary or judicial process and faces difficulties to break free from it. In practice, the disciplinary or judicial process is constituted of two to three successive phases: the problem to identify and to find, the collection of evidence for proving the problem and eventually the trial in case of appeal by the athlete (Figure 1) (7).

Figure 1. The three phases of the traditional judicial process in anti-doping (7).

The reliability and integrity of the whole process are guaranteed by the very strict quality management system offering a complete follow-up and recording of the chain-of-custody from sample collection to the reporting of adverse analytical findings, and further on to their presentation in court if the athlete appeals against the results of anti-doping tests. However, while this process is continuously improving, the efficiency of the fight against doping should be measured according to the progresses accomplished towards the fulfillment of detection, reduction, solving and/or prevention of doping objectives and not in terms of analytical case numbers. Indeed, these concepts stated in the Code also serve important health protection purposes (3).

Noteworthy, since the 2009 revision of the Code, methods of establishing facts or presumptions of facts related to an anti-doping rule violation may be established by any reliable means. This addition is a key point as it provides a legal basis for a more flexible fight against doping. Likewise, widening the scientific horizon of the field by including an intelligence aspect might be interesting. Actually, the situations of doping and traditional criminality share a number of similarities in view of their mechanisms and the legal context in which they fit. Forensic Intelligence having brought remarkable solutions in recent years to understand criminality and/or in a security perspective, exploring this concept is a most promising, though difficult, avenue. The over-riding hypothesis is that a proactive Forensic Intelligence program allied to existing substances prohibition and laboratory analysis could significantly improve anti-doping efforts.

According to the number of abnormal blood profiles, a prevalence of blood doping of 12% has been estimated by the International Association of Athletic Federations (IAAF) for the entire population of samples collected from elite track and field athletes between 2001 and 2009 (8, 9). Interestingly, the prevalence of blood doping in male and female athletes ranged from 3-48% and 1-46%, respectively, depending on the country of origin. Similarly, a prevalence ranging between 10-35% has been stated in a survey on doping among German elite athletes in 2008 (10). However, less than 2% of the ~260’000 blood and urine samples collected yearly are reported to contain a banned substance or to highlight the use of a prohibited method (9, 11). This figure remains quite stable despite a tremendous increase of both the volume of anti-doping tests and the sensitivity and accuracy of bioanalytical methods.

Part of the explanation may be found in the perpetual sophistication of doping techniques in order to remain undetectable by Accredited Laboratories, the seemingly constant flood of and ease of access to new medications with doping properties, the latency between their marketing and inclusion to the List and the random nature inherent to out-of-competition (OOC) anti-doping tests. Despite the most sophisticated doping methods being the prerogative of elite athletes almost exclusively, doping with new and unregulated substances may touch each and every category of athletes.

In recent years, the practice of doping has evolved towards continuous micro-dosing and the mixing of a cocktail of designer drugs to achieve the desired performance-enhancing effect (12). Contrary to punctually using a single substance in high doses, this methodology offers the advantage of resulting in concentration levels that are difficult to detect, being near or below the limit of detection (LOD) of the methods. Indeed, as illustrated by the Marion Jones and Lance Armstrong cases in 2007 and 2012, respectively, athletes with medical advising on intelligent doping may remain undetected throughout their careers despite countless anti-doping tests (13, 14). The introduction of OOC anti-doping tests in the early 1990s in response to massive steroid use during training periods throughout the 1980s was an enormous paradigm shift in the fight against doping (15). Nevertheless, while complementary to IC tests and a major improvement in the deterrence of doping ever since, the efficiency of OOC testing is facing organizational, economical and scientific challenges. Testing every single athlete on a regular basis would be unrealistic and completely out of proportion considering the costs, the volume of samples to analyze and the conflicts with the athletes’ privacy it would generate. In this situation, refining the targeting of athletes is a necessity, but the increasing complexity of doping makes it difficult to highlight likely cheaters and to test these individuals timely before complete wash-out of doping agents. As a result, and with the exception of the Athlete Biological Passport (ABP) helping in the targeting of blood doping, OOC tests are still

tainted with randomness. Thus, only the tip of the iceberg seems to be perceived with today's approach of the fight against doping.

Regarding this topic, the use, or attempted use, by an athlete of a prohibited substance or a prohibited method is only one of the eight rule violations described in the Code (3). Possession of prohibited substances or methods, trafficking or attempted trafficking of any prohibited substance or method and administration or attempted administration to any athlete in-competition of any prohibited method or prohibited substance also constitute rule violations. Nevertheless, only a small research effort has been dedicated to investigating on all these regulatory breaches, leading to succinct scientific approaches of limited exploratory nature. In the light of the global scope of the Code when considered as a whole, the fight against doping has to multiply the perspectives upon which such a complex problematic is addressed. Indeed, considering the growing number of studies indicating the existence of doping networks or team-organized doping and a grey/black market for prohibited substances, these aspects deserve deeper examination and the elaboration of specific strategies (16-19). The Bay Area Laboratory Co-Operative (BALCO) case which occurred in 2003 in San Francisco, the Operation Puerto which took place in 2006 in Spain and the Lance Armstrong case that came to light in 2012 illustrate these particular concerns. To date, the investigations conducted during these prosecutions remain quite unique in their kind, regardless of the magnitude of doping that they revealed.

To some extent, the current situation results from an allocation of human and temporal resources having to respond to scientific, legal and organizational needs and challenges. On a daily basis, WADA Accredited Laboratories are absorbed by the constant and significant flow of blood and urine analyses required by National and Regional Anti-Doping Organizations (NADO and RADO), National and International Sports Federations (IFs) and the International Olympic Committee (IOC) and the regular demands for case support. Additional concerns, related to research and the challenge of developing and validating methods for the analysis of newly prohibited substances and methods, as well as proficiency testing programs, internal and external quality management audits, ISO 17025 accreditation surveys and the specific requirements of the ISL complete the schedule (6). These different aspects naturally constitute a priority for the Accredited Laboratories in the accomplishment of the mission entrusted to them.

David Howman, WADA's Director General since 2003, declared about it (12):

"Testing is – and always has been – the bedrock of the fight against doping in sport; science being used against science, with the hope that 'our' science – that of the anti-doping community – one day becomes too sophisticated for athletes to risk doping."

This statement reveals a perception of anti-doping's practice where most of the burden of the fight has been set firmly on the shoulders of Accredited Laboratories with hopes of solving the problem by decisive improvements in screening methods.

This framework promotes perpetual and endless technological evolution to detect, identify and quantify every prohibited substance/method appearing in the List. However, due to the operational aspects previously described, researchers and other partners of the fight against doping are bound to focus on the disciplinary or judicial process rather than making room for thinking outside of the box. As a consequence to this case-by-case and justice-driven approach, anti-doping is still missing a strategic vision to understand the phenomenon and to propose long-term solutions. At present the resources to gain timely and relevant knowledge to prevent and interrupt doping are limited and the field concentrates its efforts on solving isolated cases.

In parallel, this tunnel vision induces undesirable side-effects on court proceedings themselves in case athletes appeal against adverse analytical findings. Depending on the general composition of the panel in scientific matters, influence of perceived more than objective science can be significant and ultimately influence the overall decision of the panel. Due to this difference of perspective between scientists and lawyers, the anti-doping judicial process is often perceived as a discussion on the abilities of the scientist rather than the value of the analytical results themselves and leads to their erroneous or partial interpretation. Assessment of an anti-doping rule violation has to remain in the hands of the judge, as the only entity able to weigh every piece of information in the light of each party's plea. However, where scientists should be presenting analytical results in a balanced fashion regarding a potential doping offense to bring scientific support to a case, the judge and lawyers request a personal opinion on the significance of an Adverse Analytical Finding (AAF) highlighting the presence of a prohibited substance or its metabolites or markers in a biological sample. This common practice is problematic and may result in a disputable alteration of the legal process. Indeed, while scientists in court are usually clearly identified as parties or as independent scientists, scientists may become advocates for one side and may unbalance the legal outcome of a trial as the original definition of their roles is sometimes challenged and difficult to respect (20). Due to the blur around this aspect of the use of science in the legal system, lawyers seek for procedural irregularities or legal flaws as a pretty straight-forward and efficient way to challenge and to rule out the validity of the technical expertise of Accredited Laboratories. The testing procedure as a whole is disputed, from sample collection, transportation and storage, to chain-of-custody considerations and the very bioanalytical process itself from start to finish (21). Since evidence gathering is usually limited to the collection of a single AAF, its value is decisive to the outcome of the arbitration. Nevertheless, as it comes most often as a single element during court prosecution, trying to find any deviation from the rules during the testing procedure has become a regular aim for lawyers to weaken or even

invalidate the analytical result (21). As a consequence, the whole process shows insufficient emphasis on the physiological meaning of an AAF as the attention of the court is diverted from its original purpose, which may bias the whole process. Under the principle of strict liability, the burden of proof is for the athletes to defend themselves. Indeed, this rule states that *"an athlete is responsible, and an anti-doping rule violation occurs, whenever a prohibited substance is found in an athlete's sample. The violation occurs whether or not the athlete intentionally or unintentionally used a prohibited substance or was negligent or otherwise at fault."* (3). In view of these circumstances, finding legal flaws is a prime and more vulnerable target for the defense which has restricted room for action and more often than not very limited scientific expertise. In order to guarantee more fair and balanced court proceedings, an important step towards a more comprehensive evaluation of AAFs and their relationship to doping could be initiated by collating products of intelligence that originate from broader sources of information.

A further illustration supporting this view may be found in the concept of threshold and non-threshold substances, where substances are prohibited either above a urinary threshold concentration, or so-called cut-off, or regardless of the concentration. If the urinary concentration of a xenobiotic is significantly above or below the threshold, the evaluation of a potential doping offense is eased, but the closer to the limit, the more complex and the more subject to court disputation. Assessing the origin of a non-threshold xenobiotic detected at a very low concentration level is equally difficult. Considering the analytical uncertainty and potential bias associated with qualitative and quantitative results and potential sources of issues such as sample contamination or degradation due to freeze-and-thaw cycles and/or bacterial activity, the qualification of a suspicious sample as an AAF by the laboratory and the decision to report it as such may turn into a black-and-white process. In parallel, as the sensitivity of bioanalytical tools has significantly improved over the last decade, with a detection capability in the pg/mL range for more and more substances, assessing the origin of low concentration levels and the question of the dose-response effect on performance become increasingly difficult (15, 21). A first attempt at addressing this problematic may be found in the introduction of MRPL for stimulants, narcotics and β-blockers from IC samples in 2009 (22). These concentration limits, either defined at the MRPL itself or % of the MRPL, were established as safety margins to determine whether a suspicious urinary finding is due to deliberate exposure to a doping agent or inadvertent environmental contamination. This concept originates from a case where experts faced difficulties in determining if the trace quantity of a prohibited substance detected in urine was linked to active consumption several days before the competition or to recent exposure in daily life (21). Noteworthy, the lack of performance-enhancing effect in very low concentration levels was also pointed out, bringing additional support to the introduction of MRPL. Ever since, the technical document has been revised, including floor values at 50% of the MRPL. The

idea was to reduce the MRPL while maintaining similar floor levels for reporting non-threshold substances prohibited IC only (21). Actually, WADA recognizes individual capabilities for some laboratories in their ability to identify a wider range of prohibited substances or lower concentrations than other laboratories. Therefore, MRPL have been established as a minimum concentration that laboratories must be able to routinely detect and identify to ensure that all Accredited Laboratories report results in a uniform way and operate at the same minimal level (22). Since these values are neither a threshold, nor a limit of detection, AAFs may result from concentrations below the established MRPL. Nevertheless, in practice they may be perceived as additional cut-off limits sharing similar concerns as previously mentioned for threshold concentrations.

Therefore, the use and strict compliance to threshold concentrations and MRPL may be questionable as this induces a “leap of faith”, which demonstrates pernicious effects on anti-doping (23). Accredited Laboratories may be tempted to develop a habit of not reporting AAFs close to the threshold limit or under the MRPL to avoid any disputation of their performance in court or simply to be facing difficult cases. Actually, this practice is an involuntary outcome of the lack of intelligence use, which creates a substantial information gap and complications around the interpretation of suspicious findings. A framework of circumstantial information related to the case, the individual physiological variability of the athlete under scrutiny and scientific studies addressing the consumption of the incriminated substance should be considered more systematically during the interpretation of an AAF. Indeed, a tendency may develop to report results within a certain zone of comfort rather than within concentration ranges needing a thorough interpretation of the physiological meaning or prone to questioning on the analytical work’s reliability.

I.1.4. Recent doping cases

A controversial incident occurred in 2003 when three times Track Cycling World Junior champion Mark French was alleged of prohibited substances use and supply and/or trafficking in the months before the Olympic Games in Athens (24, 25). Traces of glucocorticosteroid were discovered in used drug syringes, needles, empty vials of equine growth hormone and vials of a homeopathic compound found in a rubbish bin in his room at the Australian Institute of Sport cycling training facility. While CAS was unable to prove Mark French’s use of growth hormone, he received a two-year ban from competing and a lifetime ban from the Olympic Games. After the first hearing, Mark French appealed the 2004 CAS decision. The panel eventually concluded that there was insufficient evidence to confirm beyond reasonable doubt his use and supply and/or trafficking of growth hormone, and insufficient amounts of glucocorticosteroid in the homeopathic compound to have a significant physiological effect (24, 26).

This incident illustrates common issues in the collection and presentation of evidence in court that anti-doping authorities are facing when addressing a wider range of rule violations. Indeed, despite the seizure of prohibited substances and injection material in Mark French's room, the appeal procedure was successful. This case demonstrates the necessity of a different approach for the investigation on doping offenses in the absence of AAFs: forensic science methods may be a good answer.

A more recent example where the handling of injection material followed an appropriate methodology for this type of evidence may be found in the 2007 Rowing World Cup case (16) (original paper in Appendix V.2.3). During the competition, a plastic bag containing medical equipment was discovered in a waste container. Considering the probability that elite rowers may have used this equipment, the International Rowing Federation (FISA) assigned LAD with the investigation. During the initial phase, the substances were identified as non-doping agents. However, the use of an intravenous system is an anti-doping rule violation and syringes, needles and used perfusion material were also found alongside these products (3). Therefore, the traces of biological material visible inside the infusion tubing were collected for DNA profiling to identify the source donors. With the help of contextual information and the information provided by the physical examination of the packaging of the seized items, the list of athletes to target could be narrowed down to a few individuals, whose DNA profiles were determined after the collection of blood samples serving as reference material. A match between the genetic markers of the traces and the reference DNA profiles provided evidence on the use of a prohibited method by 8 different rowers (16). Accordingly, the FISA hearing panel sentenced the athletes with a two-year ban from competing but also the coaches and officials of the National Federation from any future involvement with competitive rowing (27).

The BALCO incident is a more extensive and complex example of the use of police investigation methods. Prior to the beginning of the Federal investigation in 2003, an anonymous source provided the University of California Los Angeles (UCLA) Olympic Analytical Laboratory with a syringe containing a designer anabolic androgenic steroids (AAS) unknown to anti-doping authorities, which they named tetrahydrogestrinone (THG) (28). Trevor Graham, a sprint coach whose clients included Marion Jones and Tim Montgomery, was later identified as the anonymous informer. When the laboratory owned by Victor Conte was raided by the U.S. Internal Revenue Service Criminal Investigations Unit and the San Mateo County Narcotics Task Force Records in 2003, large quantities of doping agents of all kinds and materials related to the activity of the laboratory were seized (29). According to the records, Patrick Arnold, a chemist, was responsible for the design of THG and proposed BALCO to distribute it. The investigations revealed that numerous top level athletes were listed in the BALCO files, including Marion Jones (three times Olympic gold medalist and twice silver

medalist), Tim Montgomery (100m world sprint record holder), Barry Bonds (baseball homeruns record-breaking hitter) and Kelli White (100m and 200m world sprint champion) (30). This extensive investigation resulted in the effective prosecution of several athletes for doping offense in the absence of any adverse analytical chemical evidence, which was an unprecedented event in the history of anti-doping (29). An interesting outcome of this successful collaboration between the United States Anti-Doping Agency (USADA), the Internal Revenue Service Criminal Investigations and law enforcement agencies was the recognition of other reliable sources of information for identifying and proving a doping case. This legal notice was subsequently included to the 2004 revision of the Code (31). Noteworthy, as this scandal raised awareness on the widespread use of doping agents and the limitations of the anti-doping legislation in American professional sports, an improvement of dope testing programs in the U.S. quickly followed and was directly supported by the White House.

Similarly, in 2006, the Operation Puerto shed light on the widespread doping network of Dr Fuentes, the former Kelme cycling team physician, involving blood doping and transfusion, steroids and growth hormones intake, etc. (32, 33) The case arose after the confession of Jesús Manzano on systematic doping during the years he spent cycling under the medical supervision of Dr Fuentes. As doping is an offense under the Spanish penal law, the Guardia Civil conducted an investigation and seized 200 blood bags and 105 drugs and doping agents (testosterone, human recombinant erythropoietin, growth hormones, insulin,…) at the office and home of Dr Fuentes. Furthermore, a training and doping calendar containing a coded list of approximately 100 names was discovered, which included a majority of top level cyclists and pointed out serious suspicions of blood doping on a number of professional tennis and football players. By overlapping this information with the hematological follow-up of professional cyclists, a few athletes who had previously won the most prestigious races in cycling (Tour de France, Vuelta, Giro, Pro Tour, Olympic Games, etc.) could be identified and convicted of doping. While the collaboration between sport authorities and the Spanish Police in leading the investigation was successful and promising, their joint efforts faced reluctance from the political world to shed light on the rest of the athletes appearing on the list.

In 2012, the USADA vs. Lance Armstrong case further illustrated the invaluable role of criminal investigations in detecting highly professionalized organized doping. Despite the lack of direct bioanalytical evidence, the USADA’s investigation was successful in collecting sworn testimony from 26 people, including 15 riders with knowledge of the US Postal Service Pro Cycling Team doping activities, as well as 11 teammates of Lance Armstrong. In particular, 9 of his teammates were also clients of the Dr Michel Ferrari who supervised the doping program among the cycling team. The evidence collected during this case covers the entire career of Lance Armstrong since 1998 and are of documentary, scientific, direct and/or circumstantial nature. Indeed, the investigation allowed gathering a multitude of banking and accounting records reflecting more than one million dollar in

payments to Dr Ferrari and extensive email communications during a time period in which Lance Armstrong claimed to not have a professional relationship with his doctor (13, 34). Furthermore, a vast amount of additional data have been collected, including laboratory blood test results throughout his career and the scientific expertise realized afterwards to interpret these data from a longitudinal perspective. These elements as a whole allowed demonstrating in a univocal way the use, possession and distribution of performance enhancing drugs by Lance Armstrong. According to the USADA, *“the US Postal Service Pro Cycling Team ran the most sophisticated, professionalized and successful doping program that sport has ever seen.”* (34). Under this program, Lance Armstrong would not only win the Tour de France seven consecutive times between 1999 and 2005 to set an unprecedented record in cycling’s history but would also not be tested positive.

I.2. Rationale and hypotheses on areas for improvement

These examples illustrate a tactical use of information to provide investigative leads and to feed the judicial process. However, these cases would hardly have come into scrutiny without confessions or denunciations and show the lack of a rationale and model for gathering relevant information and detecting doping issues. Therefore, a more systematic and in depth use of data appears necessary to favor a proactive detection and identification of suspicious activities. In order to truly deter athletes, the timely dimension is of outmost importance. Indeed, the impression that only the unlucky or most obvious ones get caught may naturally survive as long as a rule violation is discovered only years after the athlete having enjoyed all the fame and glory of past victories.

As a result, fight against doping needs to consider other scientific avenues to understand and highlight doping offenses so as to exert efficient preventive or disruptive effects and to support educative or policing decisions by regulatory bodies. The opportunity to gather broader, yet equally relevant, sources of information should be taken in order to understand doping and its countless variations and to support cases or situations that need action whether in reducing a risk, preventing some substance use, legitimating observed results or leading to punishment by courts. Anti-doping needs scientific innovation rather than technology innovation. Indeed, performance of complex analytical chemistry and biochemistry tools and WADA accreditation of laboratories have been the center of attention whereas gathering and exploiting information coming from a multitude of sources has remained obscured by technological and quality management debates.

In this view, this thesis proposes to discuss the problem of doping and to assess the additional value of Forensic Intelligence for implementing more specific and efficient models to prevent, reduce and/or solve doping in sport. The potential of an Anti-Doping Intelligence to address the different aspects of the problem will be discussed relying on a logical reasoning model similar to the one used

to understand and impact on criminality. The phenomenon of nicotine consumption in sport through smokeless tobacco use, and more specifically snus, will serve to evaluate the extent to which processing information coming from a variety of sources may be inserted into this model.

On a side note, doping concerns the sports community as a whole and may be perceived as a public health issue. Elite and professional athletes represent only a small portion of all the sports enthusiasts worldwide. Doping among amateurs and practitioners of sports not legally bound by WADA-IOC is an equally important matter. Since this extensive part of the community is usually not subject to anti-doping controls, measuring the prevalence of doping through analytical monitoring is difficult, if not impossible, and requires questionnaires, such as randomized-response surveys. Sanctioning these sports enthusiasts is very unlikely and might be difficult to justify in most instances. Likewise, it would probably go beyond the primary scope of WADA for legal, organizational and economical reasons. Intervening on doping in this population requires the development of preventive and educational tools rather than using the threat of a sports ban to exert a deterrent effect. While the issue of doping is important regardless of the type of athlete and sport, the thesis will focus on elite athletes bound to WADA-IOC regulation.

CHAPTER II | FORENSIC INTELLIGENCE & ANTI-DOPING

II.1. Forensic Intelligence

II.1.1. General concept

Forensic science is an important aspect of the criminal justice system, where it serves to lead investigation or to support law oriented questions with scientific information. Analysis can be based on logic besides tools.

Forensic Intelligence embraces this approach and pursues a further goal in the study of criminal activities by bringing a broader logical dimension to the interpretation of trace data detected and collected after a litigious activity. The characteristics of these remnants are extracted and described before being integrated to a structured memory containing information previously acquired on the phenomenon. Afterwards, inference structures are built upon logical analysis of these data, allowing revealing a network of hypothesized links between fresh information and material already memorized. This gathering of knowledge on the criminal phenomenon under scrutiny can support decision-making of tactical, operational or strategic nature in the area of law enforcement for crime solving, reduction and/or prevention.

In practice, Forensic Intelligence endorses three levels of function, namely *tactical intelligence*, *operational intelligence* and *strategic Intelligence* (Figure 2).

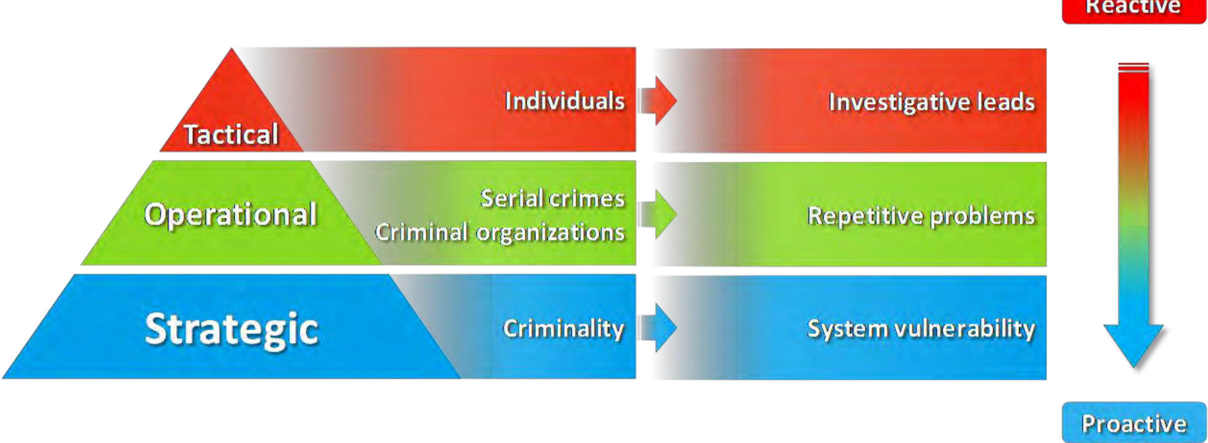


Figure 2. Organization and aims of each level of Forensic Intelligence. From the reactive micro-level of *tactical intelligence* to the crime reduction planning meso-level of *operational intelligence* and the future-oriented and proactive global-level of *strategic intelligence*.

These different levels of intelligence operate on an increasing spatial and temporal dimension. However, sharing a logical reasoning on the trace and numerous sources of information, these concepts interact rather than being completely distinct processes. Therefore, the delimitation between these different levels should be perceived as flexible due to a natural overlap.

An interesting feature is the ability of Forensic Intelligence to propose a proactive and global response for the prevention and solving of criminal activities rather than focusing on solving a single past event (35).

II.1.2. Levels of Intelligence

A. Tactical intelligence

Tactical intelligence is a reactive approach supporting real-time decision-making of front-line law enforcement officers and proposing leads to investigations at a case level. The logical analysis of traces provides accurate, timely and usable information for crime detection, for identification, localization and arrest of potential offenders, and for gathering of evidence for prosecution in court (36) (Figure 3).

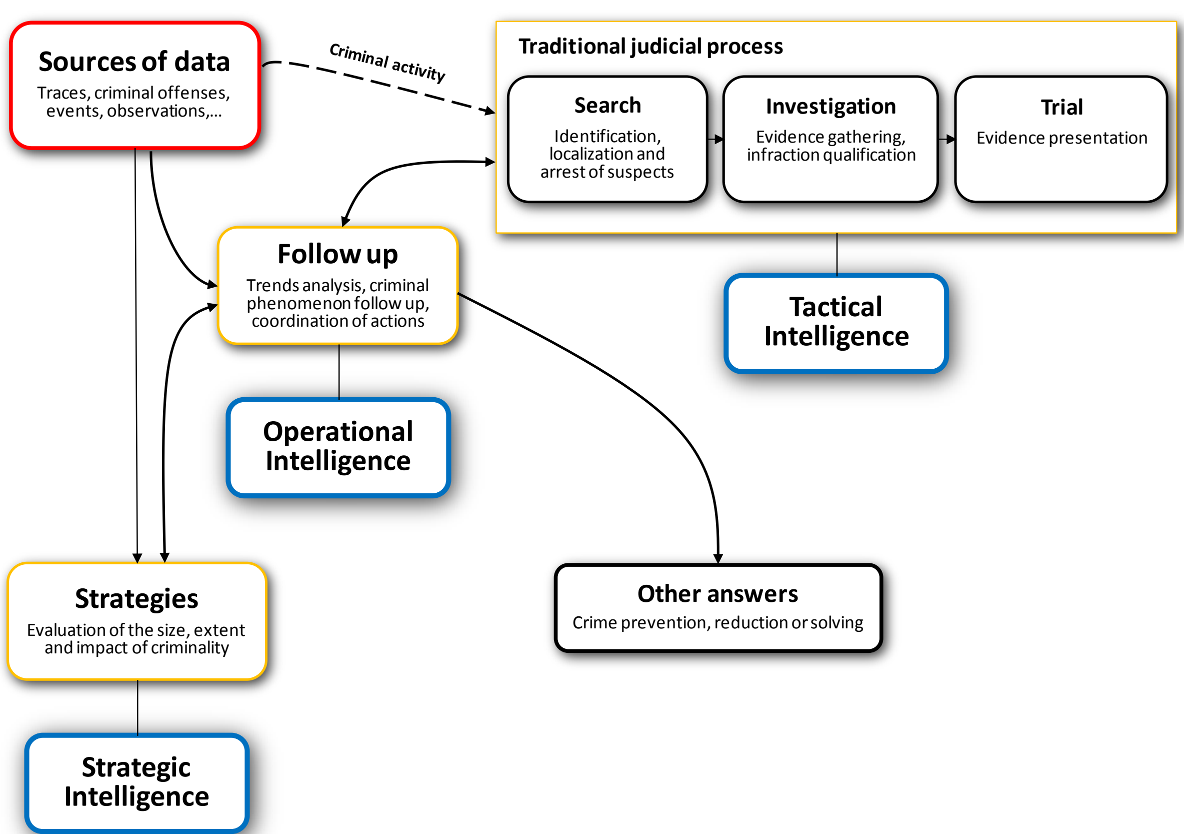


Figure 3. Structure of Forensic Intelligence through logical processing of information supporting *tactical intelligence* to feed the criminal judicial process, *operational intelligence* to plan crime reduction actions and *strategic intelligence* to develop future-oriented and long-term solutions (37).

Tactical intelligence alone does not seek to bring to light criminal activities that exist either at a scale larger than the individual offender or that constitute repetitive issues (38). The detection of such criminal problems and their subsequent solution, reduction or prevention require broader sources of information and more thorough exploitation of traces as compared to the short-term case-by-case approach of *tactical intelligence*.

B. Operational intelligence

Operational intelligence calls for a larger organization level to provide a comprehensive understanding of criminal trends, to ensure a follow up and to help in the coordination of actions

(Figure 3) (38). This concept seeks to impact on repetitive problems such as serial crimes, the activity of criminal organizations or illicit drugs trafficking by promoting a more proactive and mid-term oriented approach of problem-solving. Indeed, *operational intelligence* assists decision makers in the targeting and deployment of law enforcement resources as well as in the planning of actions for crime reduction or prevention (39).

With the help of exploratory, statistical and visualization methods related to crime analysis, extensive amounts of information saved in a structured memory are logically processed to detect geographical and/or temporal problems, to determine the type of offenders and to identify criminal patterns, or so-called *modus operandi*. As this analysis process is iterative due to the constant flow of new information filling the memory, *operational intelligence* supports intelligence-led policing with continuously refined and updated knowledge.

C. Strategic intelligence

Strategic intelligence operates at a third and more global level of organization. Criminality is a complex phenomenon constantly evolving over space and time due to changes in demographics, economics, politics, environment, etc. Therefore, *strategic intelligence* encompasses these parameters in a criminological perspective as well as sub-levels of Forensic Intelligence. The reasoning here is a multivariate approach aiming for the description and understanding of the mechanisms behind criminality as part of an environment in perpetual change (Figure 3) (38). *Strategic intelligence* is future-oriented and resolutely proactive as it intends to foresee the development of potential or emerging criminal threats by identifying and resolving system vulnerabilities proactively. An interesting feature is the ability to identify areas where policing and harm minimization actions may achieve positive results (35).

This approach seeks to impact on the phenomenon as a whole rather than on specific criminal activities. *Strategic intelligence* can help designing most efficient strategies to restrain identified problems. This concept is conducive to proposing long-term problem-solving policies as well as preventive and educative actions or programs. Due to the nature of the goals pursued, *strategic intelligence* might also take a political dimension.

II.1.3. Structured memory

Forensic Intelligence is embracing a number of scientific disciplines to detect, collect, describe and compare traces related to litigious activities, which once collated with other sources of information and exploited using crime analysis tools provide a better understanding of criminal patterns. Considering criminality as a global phenomenon rather than isolated cases, the reasoning

does not limit to the study of physical, biological or chemical evidences to answer source and activity questions and to support decision-making at tactical, operational and strategic levels.

Actually, the operation of Forensic Intelligence depends on a structured memory of traces and information to gather knowledge and produce intelligence on a criminal phenomenon. As an organized repertoire of systematically and continuously updated or possibly applied inferences reasoning, the memory represents the knowledge we have at a certain time about the criminality under consideration: current criminal problems or patterns, serial crimes, cases linked, etc. (Figure 4).

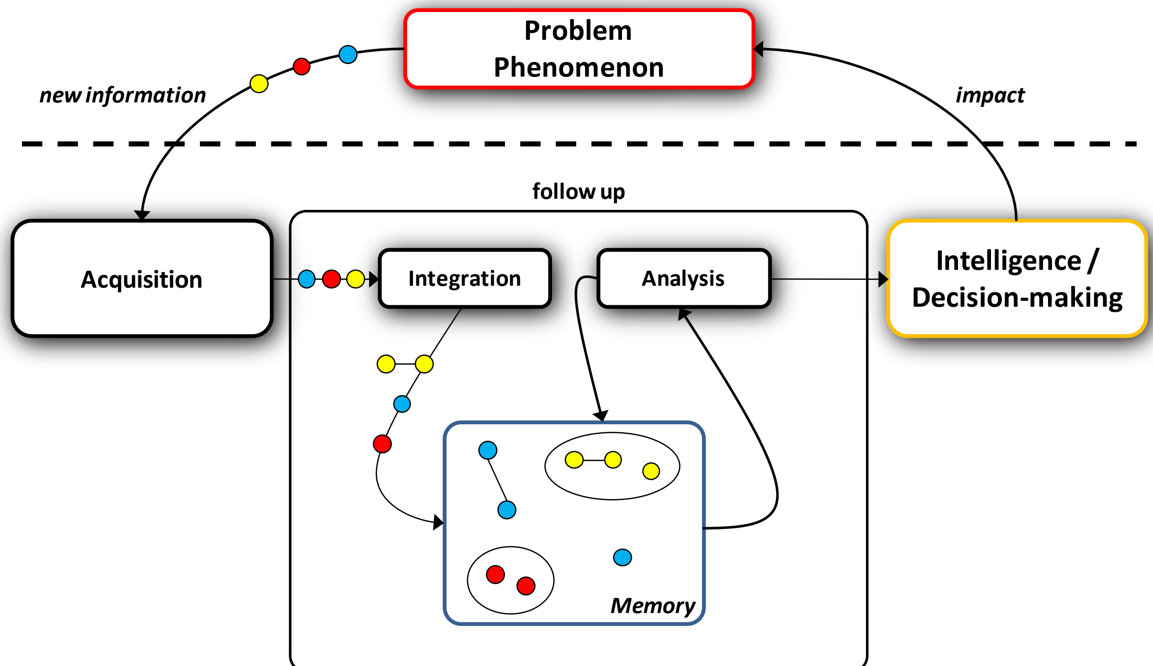


Figure 4. Logical processing of new information acquired and integrated to a structured memory containing previously stored information (40). The analysis is an iterative and cyclic process that allows transforming these raw data into timely and usable information to support decision-making in order to impact on the criminal phenomenon under scrutiny.

In practice, information on a criminal phenomenon is first acquired and then integrated into a follow up framework. Characteristics of these new data are extracted and sorted out prior to being merged with previously memorized information, allowing drawing inference structures that reveal a network of hypothesized links. This reasoning process is entirely based on postulating and testing assumptions on the relation between items organized in the structured memory.

The analysis of the memory is an interpretative step where each new piece of information may confirm the predicted truth-value of hypothesized links and/or result in the connection of sets of information originally considered as distinct. Conversely, the outcome of this logical process may question or even exclude the existence of links previously assumed. Depending on the perspective, new pieces of information may serve for immediate use or may be stored to feed future inference

processes. These new items may be organized in a short-term memory for direct exploitation on a specific criminal case, or may be integrated to a long-term memory to depict criminal trends or series or to identify system vulnerabilities.

Therefore, the follow up process is iterative and dynamic as organizing and scrutinizing the memory in order to infer new information is most likely going to modify the picture of a phenomenon and to result in an update of the memory (Figure 5).

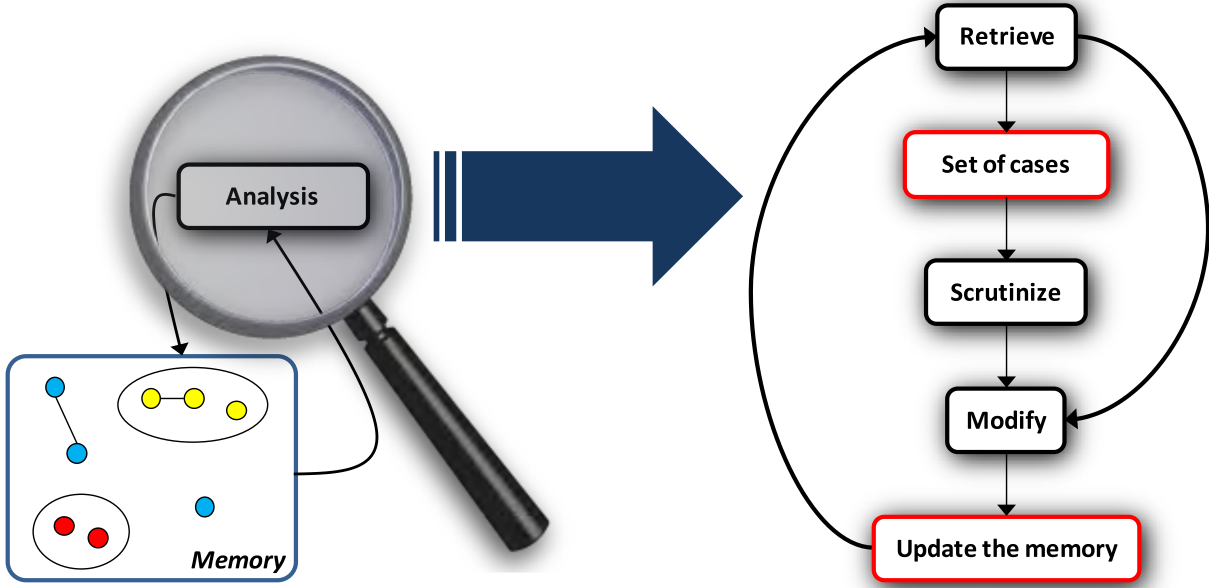


Figure 5. The logical analysis cycle of the information stored in the structured memory (41).

The coherence of the memory is ensured by this logical methodology. An interesting outcome is the ability to synthesize a set of information describing the phenomenon under scrutiny. This reasoning process provides decision makers with a real-time insight into criminality and accurate and usable information to conduct investigations, to help in the coordination of law enforcement actions and in the designing of policing operations. In case the problem has not been completely solved by these actions, but also due to environmental variations or the attractiveness of alternative targets, isolated authors as well as criminal organizations may have to adapt their *modus operandi* in order to survive and to pursue their activities. This shift in criminality requires the acquisition of new information to feed and refresh the Forensic Intelligence process in order to ensure up-to-date and exploitable knowledge. Therefore, the reasoning works in a cyclic fashion.

On a practical note, while finding links may appear somewhat straight-forward, linkage blindness may occur due to the increasing complexity of the memory, the incompleteness of the data and constraints of political, legal or organizational nature (41). Indeed, as the structure of the memory becomes more sophisticated with its expansion, the network of links may become a blur and links between objects, individuals and/or events may be missed. As a way of reducing the

splitting of information, the memory as a whole or as sets of smaller size may be visualized using relational diagrams or maps (Figure 6).

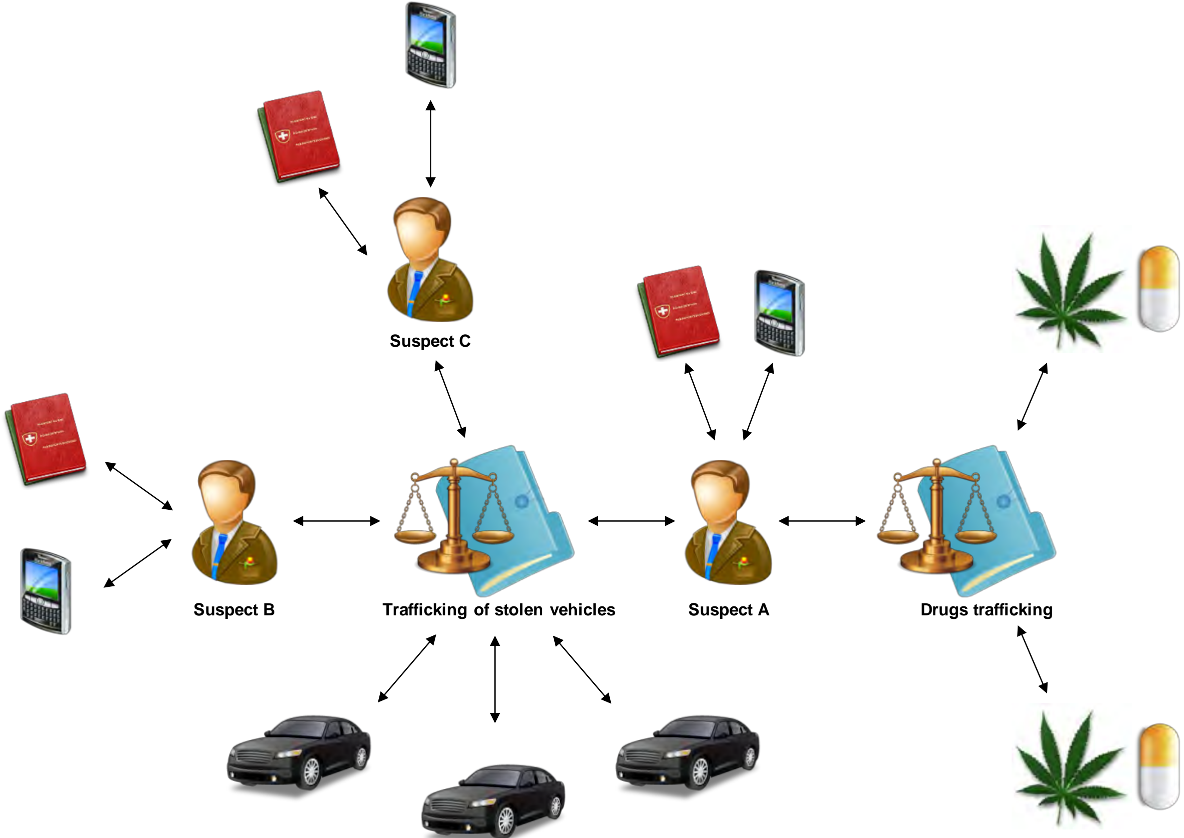


Figure 6. Simplified example of a relational diagram to visualize the links between objects, individuals and/or events related to criminal activities such as drugs and/or stolen vehicles trafficking.

Noteworthy, the concept of intelligence is a particularly versatile approach, as illustrated by its applications in the economical, sociological or demographical fields.

II.2. Perspectives for Anti-Doping

II.2.1. Transposition of the concept

The concept of Forensic Intelligence previously described may be transposed into Anti-Doping Intelligence by relying on a similar methodology and shifting the paradigm to the problem of doping in sport (Figure 7).

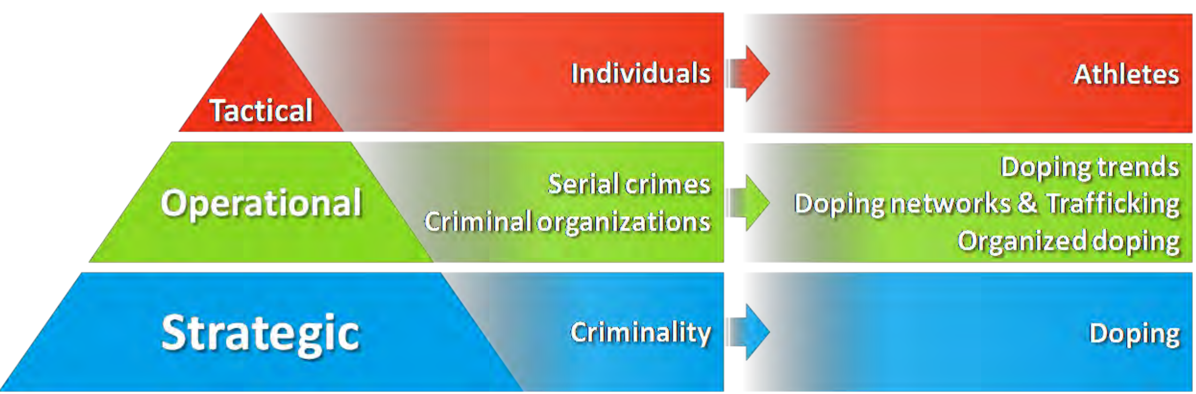


Figure 7. Transposition of the Forensic Intelligence concept into Anti-Doping Intelligence.

At tactical level, where Forensic Intelligence is focusing on the individual offenders, Anti-Doping Intelligence would be focusing its attention directly on the athletes. The exploitation of traces, including bioanalytical results, documents linked to the practice of doping and the distribution, administration and/or consumption of doping agents, seizures of prohibited substances, etc. would follow the perspective of proposing investigative leads on athletes and/or individuals responsible for the diffusion of doping as well as bringing scientific evidence to legitimate punishment from anti-doping authorities or to support the anti-doping judicial process in case of appeal.

Likewise, at operational level, the attention on serial crimes and criminal organizations would switch to tendencies in the abuse of prohibited substances, organized doping and the trafficking of doping agents. Seriality might be defined as the recurring character of these doping activities or linked to doping. The use of hormones and/or peptides represents a form of systematic doping as the methodology involves continuous intake over a long period of time rather than a single intake during a competition. Blood doping with human recombinant erythropoietin (rEPO), whose abuse began in the 1990s and shows successive waves of use each time a new generation of the therapeutic medication enters the market, also illustrates a form of seriality. Likewise, the continuity and extent of AAS abuse since the 1980s as well as their trafficking set on the worldwide scale are repetitive problems. As an example, state doping in Eastern Europe was organized systematically by governments in a quest for international recognition as opposed to their somewhat lower economic and scientific performances (1). Although this practice was characteristic of all the satellite countries of the Soviet Union, the German Democratic Republic (East Germany) also used this strategy to be recognized as a state in its own right after the division of Germany at the end of the Second World War. This relatively small country achieved incredible sport results at top level, which were acclaimed worldwide, creating a strong image despite a negative political and economical situation. AAS and other synthetic products developed in secret research laboratories to practice systematic doping are largely responsible for these extraordinary sports performances and for having helped to maintain the myth over decades. The actual situation of sports physicians supervising organized

doping programs, from import, even sometimes production, to administration of products and monitoring of the athletes' physiological values to remain undetectable is a similar issue. The exploitation of traces would serve to detect these problems, to assess their temporal and geographical dimensions and to identify the *modus operandi*. Expanding the knowledge on doping or related activities should result in a better understanding of the structure of the phenomenon and its mechanism of operation. Regarding this topic, script analysis is an innovative way to study complex forms of crime and to design more efficient strategies of action (42). This concept considers the commission of an illegal activity or any rule violation as a modus operandi rather than as a single event. Therefore, scripts analysis intends to map the complete sequence of actions and decisions before, during and after a rule violation to identify its key stages and to propose a fuller range of possible intervention points. By revealing potential weak spots where the commission process might be disturbed, disrupted or even prevented, this methodology seeks to reduce the opportunities and resources and increasing the risks associated with the rule violation (42). As scripts analysis can be retrospective, studying the organized doping cases mentioned previously would be invaluable in order to identify and counter the mechanisms underlying this particular form of rule violation.

Eventually, at strategic level, the emphasis would be put on understanding the doping phenomenon as a whole instead of criminality. The reasoning would seek to determine the predisposing factors of doping initiation among the sports community, whether they might be human, environmental, economical, or even political. With an extensive comprehension of doping mechanisms, the strategic dimension of intelligence might allow preventing the phenomenon by proposing solutions aiming at minimizing, if not neutralizing, the influence of the predisposing factors.

Considering the significant gap between the estimated prevalence of doping in elite sports and the annual WADA statistics on AAFs, the opportunity to provide a more in-depth exploitation of information at disposal or carried by traces should be seized. A series of perspectives for Anti-Doping Intelligence will be discussed in the following paragraphs to illustrate these concepts.

II.2.2. Organized doping and trafficking of doping agents

A. Overview

With the 2013 WADA Prohibited List being estimated to cover approximately 700 substances, trafficking of doping agents is an attractive and lucrative business of probably underestimated dimension that may follow complex pathways and involve criminal organizations (4, 5, 17).

Supply sources may vary quite notably depending on the type and legal status of a substance. Indeed, pharmaceutical preparations containing doping agents such as pseudoephedrine can be

readily available over the counter and require no prescription. In contrast, pharmaceutical products for therapeutic use, like rEPO or human growth hormone (HGH), require a prescription or may also be counterfeited by clandestine laboratories or diverted from production stocks of the pharmaceutical industry and supplied on the grey-market (unofficial and usually unauthorized by the original manufacturer) (29). Therefore, both original and counterfeit doping agents are being trafficked. In any case, a number of illicit products sold on the black-market (underground illegal market) by drug dealers, such as cocaine or amphetamines, are also doping agents.

In consequence, doping turns out to be a complex phenomenon considering the vast variety of substances, supplied through both legal and illegal trading routes, and the extensive connections between the people involved in these distribution networks. While doping may appear as the doing of an athlete on its own, it always involves one or several entities in the supply of doping agents or in their use, whether the friends and relatives of the athlete, or the medical staff, manager and teammates of a sport's team, or chemists, biologists and pharmacists, or pharmaceutical industries and clandestine laboratories, or criminal organizations, drug smugglers and dealers (Figure 8).

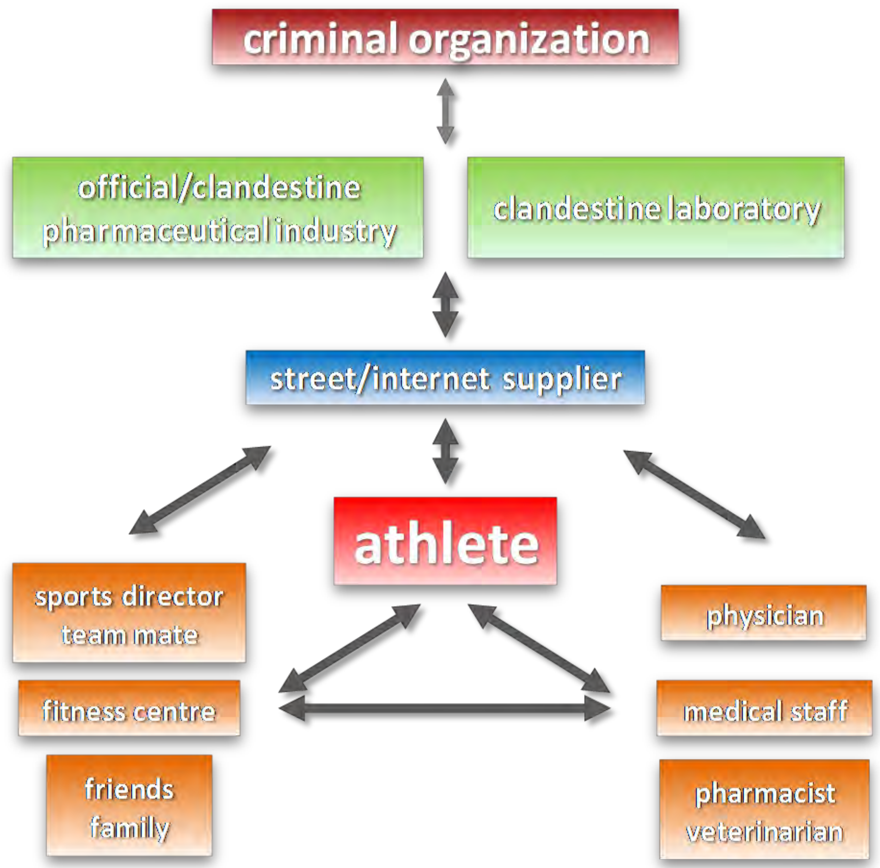


Figure 8. Doping network and the diversity of potential links between its different entities.

Since there is no regulation on the production and trafficking of doping agents in most parts of the world, there are very fertile grounds for the development of clandestine pharmaceutical plants.

On the whole, industries in Thailand, China, India and Russia play a major role with numerous production sites and well-established trading routes (17). Actually, statistics on seizures of doping substances indicate industries in China and India as the fastest growing source of suppliers of the international grey-market. The numbers reported are also corroborated by their ever-expanding pharmaceutical industries. On a global scale, producers in Thailand, China, India, Russia, Greece and Mexico account for approximately 55% of the distribution of doping agents. Besides, a hint on the involvement of the industry in this business may be found in the overproduction of rEPO, which is estimated at about five to six times the real worldwide treatment requirements. Indeed, a panel of experts supported by governmental institutions pointed out a significant imbalance between the production of pharmaceuticals with doping properties, namely rEPO, HGH and testosterone, and the therapeutic requirements of patients (17).

According to the statistics, the production of AAS might be estimated to reach approximately 700 tons a year, which is enough to supply 15 million people annually. In particular, approximately 70 tons of testosterone are produced to meet the requirements of 1.5 million people each year. Similarly, rEPO and HGH production reach approximately 34 million phials a year, enough to supply up to 2 million people (17). The diffusion of doping, which accounts for approximately 15.5 million people, does not concern only top-level athletes, but also various categories of individuals. As mentioned in the Donati report on World Traffic in Doping Substances, doping as a whole is constituted of approximately 35-37% athletes of all levels, 38-40% body-builders and gym-goers, which include private surveillance agents, 4-6% military and police forces, 1-2% people involved in show business and 15-20% false treatments (17). According to a recent survey conducted in the Netherlands, approximately 8.2% of fitness centre members use performance enhancing drugs (43).

The Internet has brought new horizons to the trafficking of doping substances in simplifying and securing its global development. The lack of regulation on the production of doping agents in most countries of the world and the multiplication of online “pharmacies” ensure the ease and safety of this grey-market, which translates into a steady growth. Indeed, there is significantly less risks for the producers and traffickers to leave the stocks in the country of origin and to send them by postal service in small quantities than to load a truck with large quantities and undertake a journey in the open through several customs to reach the final destination.

Several different forms of trafficking have been identified (17). The traditional movement of doping agents consists in loading large quantities onto a conveyance for transportation to intermediate locations, where a part of the shipment is delivered, while the rest continues to the next destinations. This form used to be the most popular previous to the e-commerce era and is becoming less frequent nowadays. A more common form, which is similar to the traditional one, relies on the postal service system for final dispatch after transportation to the intermediate

location. The advantage is to allow the gathering of different doping substances in a specific place to build up online "pharmacies" which will send small packages to the purchasers. Eventually, the most straightforward and fastest growing form involves stocking doping agents directly on-site or in the production country prior to their shipment to the online purchasers.

Although the supply of doping substances may seem to be set on the individual scale of the athlete, its major dangers and risks lie in organized doping and in the market largely linked to and dominated by international criminal organizations. The Italo-American Mafia has been in control of the trafficking of AAS and HGH in the United States up until the mid 90s and the onset of the so-called Russian Organized Crime (ROC) as a predominant actor on the grey-market (17). Soon afterwards, Asia's share into the production and worldwide trafficking of doping substances considerably expanded to replace the Russian Mafia as the leading power of the grey-market. Noteworthy, in several instances the trafficking of illicit drugs and doping agents as well as counterfeit medicines has been found to originate potentially from the same criminal organizations (44).

On a side note, the Fédération Internationale de Football Association (FIFA) and the Union of European Football Associations (UEFA) have pointed out during the Anti-Doping in Sports Consensus Meeting held at Home of FIFA in Zurich on November 29th 2013, that match-fixing and doping are linked to the same criminal organizations in many instances. Indeed, they have found an intelligence link between illegal betting and doping.

B. Intelligence and legal context

As defined by the Code, the anti-doping system is an administrative process and aims at prosecuting athletes violating an anti-doping rule. Nevertheless, the angle at which doping is addressed throughout the world may reflect differences between legal systems. Indeed, while administrative processes are the general norm and the underlying philosophy of the Code, doping in sport is a matter of public criminal law in Spain, Italy and Belgium, defining a legal framework for actions of criminal justice nature and for the use of police investigations (25). Alternatively, countries such as the United States and Australia define doping in sport as a matter of private law. Therefore, inquiries into rule violations are carried out between the IF and the athlete, prior to being transmitted to the National Anti-Doping Organization serving as an administrative body, or to a private arbitration panel, most likely the CAS. In contrast, the situation in France since the revision of its Anti-Doping Act in 2006 is a compromise between a reinforced administrative process and the criminalization of specific activities supporting the doping of athletes (45).

Pursuant to the administrative nature of the Code, and despite the awareness of the anti-doping community on the need for developing intelligent tools to link the different entities of a doping

network, the fight against doping concentrates on testing. In contrast, the use of criminal justice mechanisms in the countries mentioned previously legitimates a wider range of investigations on organized doping and taking action against people involved in doping networks. Indeed, these very specific countries consider doping as a criminal offense, which allows involving law-enforcement agencies in the anti-doping system.

Except in these jurisdictions, doping networks enjoy and live through the relative safety created by the lack of collaboration, and legal ground in the first place, which persists in sharing relevant information held at different levels by the WADA, National and Regional Anti-Doping Organizations (NADO and RADO), National and International Sports Federations, the IOC, WADA Accredited Laboratories, national customs and border agencies, the World Customs Organization (WCO), national police services, the European Police Office (EUROPOL) and the International Criminal Police Organization (INTERPOL). Indeed, these entities are as many sources of information coming from their direct contact with athletes, sports teams or sports physicians, from prevalence studies conducted by the Laboratories, customs seizures of doping agents or even police investigations where doping might be part of a criminal activity. After collection and structuring of the information, its logical processing under the principles defined in the concept of Forensic Intelligence may prove useful and particularly efficient for identifying systematic doping or trafficking networks and to neutralize, disrupt and/or prevent these activities (36). While relevant information is in possession of anti-doping authorities and their partners, implementing Anti-Doping Intelligence is facing a major obstacle in the general lack of legal regulation for exchanging data. As a key piece to the backbone of an intelligent approach, a legal framework should be settled and may be found in establishing national and international conventions to legitimate the collection and sharing of information related to doping.

With few exceptions partners of the fight against doping are non-governmental bodies, a situation which limits their freedom of action in addressing the phenomenon from a more global perspective. Ultimately, governments can decide whether to include or not the possession and/or trafficking of prohibited substances to national criminal codes, regardless of WADA's opinion. As the Code defines doping as a violation to the rules defined in this administrative regulation, treating doping as a criminal act is arguable. Nevertheless, athletes of all levels represent approximately a third of the population using doping agents prohibited by WADA, in particular AAS (17). Considering that top-level athletes are a minority bound to the Code's regulation and in most instances the only group of people under continuous medical supervision, it is obvious that the general population of users may be at high health risks. Indeed, they tend to show little knowledge or care about the products' chemistry and the prescribed daily dose and underestimate the side-effects of regular use. Since these people do not fall under the legislation of anti-doping, the trafficking organized around

this huge part of the market is also considerably facilitated. In these views, the argument of the countries previously mentioned to treat doping in a similar way to illicit drugs use and trafficking is comprehensible. One way or another, raising awareness on doping as a public health issue would help to legitimate the establishment of the legal grounds necessary to implement the different aspects of Anti-Doping Intelligence.

A Forensic Intelligence approach has been developed at the School of Criminal Justice (ESC - Ecole des Sciences Criminelles) in Lausanne, Switzerland, to help understand and fight illicit drugs trafficking as well as in the detection and description of criminal organizations involved in the counterfeiting of pharmaceuticals. Doping agents covering a large part of these categories of substances, the methodology can be described and directly transposed to anti-doping. Therefore, the term ”product“ will be used for illicit drugs, counterfeit medications and doping agents indistinguishably.

While online sales show a growing popularity, the concept of strategic internet monitoring might bring interesting information. Indeed, the use of search engines and automatic alert systems on specific keywords allows listing and following the online sales websites in order to obtain a large panorama of the market and to detect the emergence of new trends (46). The extraction of digital data on the website coding, the geographical location of the host, the contact address, etc. might indicate the origin of the products and sales areas across the world, the geolocation of the retailer and sometimes its identity. The analysis of these digital data might also highlight a link between online sales websites and, therefore, refine the comprehension of the structure and activity of the market. Applying this methodology to the analysis of forums, blogs, social networks and other online media might significantly contribute to increasing the knowledge on the activities of the distribution networks. These tools provide an overview of supply and demand and allow estimating the consumption prevalence of the substances and to follow its evolution. Physical and chemical profiling of seized products brings complementary information in order to infer the source of production.

The product itself is a trace resulting from the trafficking network and constitutes, therefore, a direct and major element for understanding the criminal organization. It carries information providing indications on source and activity decisive in the perspective of decoding the structure of the phenomenon. Drug profiling is this process of extracting the physical and chemical profiles to identify links in order to apprehend the organization of illicit drugs trafficking activities (35, 46). The optical examination of the physical characteristics of the packaging and product focuses on a multitude of parameters, including the name, logo, formulation (vial, pill, gel cap, cream, etc.), color, dimensions, batch and/or serial numbers, country of production, language and destination. These elements provide important information linked to the producer and its *modus operandi*. Following

the physical examination, active compound and excipients of the seized product are identified and measured by chemical profiling, allowing highlighting a link between similar products or, alternatively, between different products from the same production line (47, 48).

Physical examination, chemical characterization, digital data and other circumstantial data are stored in a structured memory for further exploitation. Then, inference models are built upon these data to link product seizures, highlight distribution networks and identify sources of supply. These models allow evaluating the grey and black market in order to support decision-making and prioritizing (49). Also, chemical profiling might be able to identify a potential health risk as the composition and concentration of the active compound in counterfeit medications often differs from the indications on the packaging (44).

Considering an *operational intelligence* perspective, similar chemical and/or physical profiles can bring to light a link between separate product seizures or trafficking cases that appeared unrelated a priori (35). S*trategic intelligence* can serve to visualize the organization of trafficking networks, from production to distribution, in order to identify key points and tendencies in the supply and use of illicit products. Establishing maps of the geographical zones responsible for the production of these compounds and of the consumption prevalence of specific forms of doping across the world is also possible.

The extraction of the physical and chemical profiles of doping agents seized by the customs and the strategic internet monitoring offer a global view of the market, allow identifying new medications with a doping potential, and measuring the extent and seriousness of a doping epiphenomenon. Drawing schemes indicating relations between individuals and/or substances could greatly improve the understanding of the structure of the trafficking and, therefore, the success rate of interventions aiming at tackling these activities. Actually, relational diagrams are a common visualization method to help in the process of describing the links that constitute the backbone of the phenomenon. Collection and sharing of sensitive information are essential to have a real time evaluation of the size, evolution or mechanism of a doping phenomenon. Nevertheless, custom seizures represent only a small percentage of the market for prohibited substances as the numbers of the parcels controlled when going through the customs is limited and as a seizure can be made only for products with a specific import and/or use regulation. Therefore, strategic monitoring and profiling are complementary tools.

Exploring this approach would be even more interesting in anti-doping as it may help refining the targeting of athletes or sports teams, identifying doping promoters and deploying adequate operations to dismantle core ramifications of doping networks. The overall advantage of this approach would be to elicit information of a different and improved nature compared the one

currently exploited, which may prove efficient in supporting not only tactical measures, but also operational or strategic operations.

From a practical point of view, implementing and regulating Anti-Doping Intelligence might be a difficult task due to several challenges and limitations. Since the boundaries and function of anti-doping are particular to each and every country, the harmonization of this approach might face legal obstacles. While some countries might require only little legislative amendment to implement Anti-Doping Intelligence, other countries might need substantive legal reform. Indeed, different parts of the world consider doping either as a matter of administrative law or as belonging to criminal law, with all possible degrees of variation in between. Therefore, the establishment of an intelligent system is dependent upon the will of governments, and on anti-doping authorities to press on them, to legislate over this question. In addition, centralization is essential to reduce the fragmentation of information. Indeed, despite the existence of databases such as WADA's Anti-Doping Administration and Management System (ADAMS), other at individual NADOs and even the ABP and the urinary steroidal passport at Accredited Laboratories, as well as investigation information, an organized Forensic Intelligence system is lacking. Since knowledge on doping activities is dispatched between all the stakeholders, a principal location to gather information and to operate intelligence processes, perhaps at WADA, would be necessary. This structure would help to communicate relevant and timely information with anti-doping professionals and to support coordinated actions. Again, this raises legal, organizational and economical challenges.

II.2.3. Highlighting doping with comprehensive and indirect approaches

As illustrated with the 2011 WADA/UCI v. Alberto Contador Velasco & RFEC case, the source of low-levels of doping agents, in this case clenbuterol, is difficult to ascertain due to the probability of environmental or food contamination or any other potential explanatory reason (50, 51). Indeed, due to clenbuterol's disposition in animals' edible tissues, consumption of meat contaminated with this illicit growth promoter may result in considerable issues with regards to doping controls (52). In 2010, within two days after returning from a competition in China, urine samples from a team of athletes were collected as part of regular doping controls and every specimen was reported to contain low amounts of clenbuterol (53). This situation immediately triggered the initiation of a study where urine samples were collected from 28 volunteers after returning from a trip to China. The sympathomimetic amine was detected in ~79% of the samples, indicating a widespread food contamination issue despite official prohibition of clenbuterol for animal husbandry. Likewise, FIFA initiated investigations on potential food contamination in Mexico, the host country of the FIFA U-17 World Cup 2011, after facing several clenbuterol AAFs among the Mexican national team (52). Meat samples were collected in team hotels during the tournament in addition to the regular doping

controls. Subsequent analysis highlighted a clenbuterol prevalence of ~52% in urine specimens and ~30% in meat samples.

Since similar situations tend to happen more frequently, especially due to the steady increase in sensitivity of analytical instruments, a multiparameter approach in anti-doping might become essential for interpreting bioanalytical results. The current situation illustrates the need for collaborative research between experts in the fields of analytical chemistry, endocrinology, genetics, pharmacology, physiology and sports medicine to understand and address each specific doping phenomenon and case within a forensic science framework (31). Collating analytical results, also including atypical findings and, if any, previous AAFs, and longitudinal monitoring of biomarkers with individual physiological particularities of the athlete, epidemiological, sociological, circumstantial, etc. information and products of Anti-Doping Intelligence into the structured memory may provide a logical framework enabling fit-for-purpose decision-making.

From an intelligence perspective, the structured memory attached to every athlete would help refining the targeting of suspicious athletes and identifying the likely cheaters. In addition, the memory would also serve to strengthen the use of other parameters than pure analytical chemistry results to detect and assess a potential rule violation and to support cases brought to court on appeal. Actually, when considered with other pieces of information, a xenobiotic detected at a concentration below the threshold or the MRPL might be a relevant piece of the puzzle highlighting a doping scheme. As an example, the presence of one or several exogenous prohibited substances below the threshold limit may have been repeatedly highlighted for an athlete. In this context, each individual test does not consist in a doping offense. However, considering a longitudinal perspective, this is an interesting indicator of a potentially continuous substance intake and doping behavior. Concerning endogenous compounds such as AAS, HGH, human chorionic gonadotropin (hCG) or rEPO, concentrations in biological fluids may vary naturally quite significantly, regardless of a substance intake. Indeed, the influence of genetic factors, ethnicity, sex, diseases or diet on concentration levels and/or carbon isotopic ratio ($^{13}C/^{12}C$) of AAS molecules has been reported in the literature (54, 55). Therefore, a simple concentration measurement may not be sufficient to distinguish between the endogenous or exogenous origin of the substance and assessing other biomarkers may be necessary. On the one hand an athlete may dope with an endogenous substance and yet go unnoticed due to a naturally decreased physiological response to a substance or a naturally elevated rate of metabolism resulting in a very short detection window. On the other hand, an athlete may show a naturally elevated physiological variability in the concentrations of an endogenous compound and meet the criteria of an AAF without any substance intake. The situation of the endogenous compounds underlines the need to set individual threshold limits for athletes and/or to develop indirect approaches where biomarkers and/or longitudinal monitoring would be

more informative to determine the origin of a substance and to increase the detection window. In this view, the structured memory would be an invaluable tool to record and structure the information for further exploitation and interpretation.

Accordingly, the ABP aims at the indirect detection of blood doping in the form of blood transfusion or the use of erythropoiesis-stimulating agents such as rEPO (56). The hematological profile has brought pioneer work as a first step in the development of a logical and inferential approach for the interpretation of indirect markers of doping and the determination of individual confidence intervals. Indeed, the longitudinal monitoring of multiple blood parameters has initiated an important move towards the elaboration of models to interpret data based on transparent and sound logical processes. This approach provides a more comprehensive evaluation of observing changes in levels and ratios of blood parameters if an athlete is doped rather than clean, by taking into account natural variations over time to set personalized and adaptive confidence intervals and limits for each athlete (57). As such, the likelihood of observing significant changes in an ABP due to blood doping is balanced with several potential natural causes known to modify the erythropoiesis and other blood parameters. The athlete's hematological profile allows refining the targeting of athletes by generating alerts when abnormal variations are observed. This is most helpful to increase the efficiency of doping controls and to deter blood doping, or to take disciplinary actions when an Adverse Passport Finding (APF) is declared (58).

Likewise, steroidomics, a specific area of metabolomics, aims at the discovery of biomarkers of doping with synthetic analogues of AAS or indirect doping with selective estrogen receptor modulators (SERMs), aromatase inhibitors or hCG. The steroid profile is constituted of concentrations and ratios of endogenous steroidal hormones, as well as related precursors and metabolites. The modification of this profile, due to either direct or indirect stimulation, is processed through chemometric models taking into account the global steroid metabolism to distinguish between a natural physiological condition and steroids misuse (59). In parallel with the ABP, the current development of a urinary steroidal passport integrating the longitudinal monitoring of indirect markers of steroids misuse shows promising preliminary results as a way to extend the detection window and increase the sensitivity of the tests (59-61). As a consequence, the rate of false-negative results may be greatly reduced. Alternatively, the risk of false-adverse analytical finding resulting from a single spot blood or urine test may be significantly reduced since such indirect approaches can highlight a natural cause to physiological variations exceeding the general population limits. Bearing in mind the legal consequences for the athlete and also for the laboratories keeping this risk at a strict minimum is mandatory (56).

Interestingly, the strategy behind the identification of indirect markers allows highlighting physiological variations due to doping independently from the type of prohibited substance or

method used. This aspect is particularly advantageous for overcoming the limitations of current analytical methods and of the legal system in anti-doping as well. Indeed, latency exists between the apparition of new therapeutic agents and designer steroids on the market, their detection by anti-doping authorities and subsequent inclusion to the List. Additionally, there is inertia between the annual update of the List and the development of bioanalytical tools to identify and quantify these substances. In any case, as the dissimilarity between endogenous compounds and exogenous and their exogenous counterparts for therapeutic use tend to decrease with the progresses of pharmaceutical science, along with the overall decrease in doses used for doping, direct detection is increasingly difficult.

The hematological and steroidal passports may also serve a further purpose in the evaluation of the performance enhancement after substance misuse in order to assess the degree of gravity of a doping offense, besides a general overview of the health of the athlete himself. Indeed, an athlete doping with low-levels of a prohibited substance might not show a significant physiological response, hence benefit from a limited doping effect, as opposed to another athlete involved in heavier misuse. With regards to these scientific considerations, the reasoning around doping may evolve towards more equitable and flexible justice mechanisms. Actually, compared to criminal law, the anti-doping legislation defined by the Code is somewhat rigid since it leaves very little room for a scale of penalties based on the gravity and circumstances of the offense. Furthermore, the inequalities created by this situation might be reinforced since the 3rd revision of the Code due to come into effect in 2015 contains an increase of the two-years ban into a four-years ban and reverses the onus of proof thereto. Indeed, where anti-doping authorities had to show aggravating circumstances to double the ban from two to four years, athletes will have to demonstrate lack-of-intent for a reduction from four to two years. In this context, the situation of recreational drug abuse is subject to a large debate within the community as the distinction from regular performance-enhancing substances has been abandoned in the final draft that was accepted. A unique sanction will be pronounced whether the athlete is using a product for recreative, addiction or doping purposes. Conversely, more emphasis on fair judicial proceedings might result from this significant increase in the length of the ban. Indeed, due to the gravity of the ban and its serious consequences on the life of the athlete, the situation might evolve towards a more flexible and complete evaluation of cases brought to court. Accordingly, the principle of proportionality is very likely to be accentuated. As of today, predicting accurately the practical evolution of the legal system in anti-doping is impossible.

Within a *tactical intelligence* framework, longitudinal and indirect approaches would help to support on strong scientific grounds cases of systematic doping and/or doping with substances mimicking the function of endogenous compounds. This methodology would also allow refining the targeting of athletes to improve the detection of doping. Likewise, within an *operational intelligence*

framework, collating structured memories of athletes containing their longitudinal monitoring would help in highlighting trends. Indeed, simultaneous suspicious results across populations of athletes may indicate widespread misuse of a specific doping agent or organized doping.

II.2.4. Detection and monitoring of doping phenomena

Products of Anti-Doping Intelligence may help in the detection and description of a potential, emerging or existing, yet unnoticed, trend in the consumption of doping agents. In this perspective, the information feeding the organized memory may come from a variety of sources, including sociological studies on substance use or abuse, a notice of release from the pharmaceutical industry on a new medication with performance-enhancing properties, statistics of law enforcement authorities on substance trafficking or the reporting by the medical staff of a sport's team of a growing trend in the consumption of a particular substance, etc. The exploitation of these elements stored in the memory in addition to internet monitoring, as described previously, would serve to detect substance misuse and to measure variations in the prevalence between sports disciplines, individual and team sports, national and international level, genders and also across space and time (10). Thereof, internet monitoring and all these studies, surveys and fact sheets would be very informative tools to highlight and inform on the size, seriousness and evolution of a problem. Through logical processing of these elements, the threat on all different aspects and values of a doping-free sport may be assessed and the mechanisms behind doping initiation better understood.

Actually, prevalence measurement is a recurring topic of underestimated importance and prone to analytical and logistical challenges as well as legal limitations. Doped athletes might slip through the cracks for a number of reasons, including being tested outside of doping periods or after complete elimination of the prohibited substance, or simply not being tested. Conversely, as targeting methods keep improving, testing is becoming gradually more intelligent, hence the population of athletes subject to anti-doping controls might become less representative of the general population. As a result, the statistical significance of prevalence measurements through analytical monitoring might be disputable. Then, as mentioned in the ISL, samples must be analyzed to detect prohibited substances appearing on the List and no sample may be used for any other purpose without the athlete's written consent (6). A complete removal of all identification means must be ensured before using samples for research purposes, in order to prevent any traceability back to a particular athlete. Since obtaining the consent of the athletes in the first place is extremely challenging, this legal mention makes it very difficult for exploratory epidemiological studies in anti-doping. Therefore, the discovery of trends in the consumption of unlisted or designer products with performance-enhancing properties is greatly complicated, especially as an exception to the law is always difficult to justify. Removing this rule could bring significant benefices, particularly as an

earlier detection of new doping trends and a quicker reaction could be initiated. Likewise, a more systematic use of randomized-response surveys might help overcoming these limitations and highlighting new trends. The guarantee of anonymity and impunity for the participants usually restricts data bias and improves the accuracy of prevalence measurements.

Anti-Doping Intelligence would improve the gathering of this knowledge through the multiplication and logical processing of the input sources mentioned earlier in this section. This would be helpful to feed both *operational* and *strategic intelligence* in the perspective of evolving towards anti-doping programs adapted to the specificities of each situation. Finding a unique solution to all problems appears somewhat unrealistic, whereas designing multiple and tailor-made strategies may be more fit-for-purpose. This opinion may be perceived as opposite to the principle of universality of the Code, which was fundamental to the creation of WADA in the first place and ensures the harmonization of the operation of anti-doping. Nevertheless, the limitations of tailored rules faced in the pre-WADA and pre-Code era might find a tangible explanation in a fight against doping suffering from organization and coordination imperfections at the time. Today's situation is of a different nature, with WADA bringing a clear vision and structured processes to rule anti-doping. Therefore, tailor-made anti-doping programs might offer an additional value to the fight as long as they remain consistent with the principle of universality underlying the Code. As highlighted during the 2013 Anti-Doping in Sports Consensus Meeting held at Home of FIFA in Zurich, cyclists or football players are likely to use a different mix of prohibited substances and methods to improve their performance as compared to weight-lifters for instance. Therefore, parameters such as training periods and the cultural aspect of doping in specific sports have to be taken into account when designing testing programs. Quality over quantity was advocated during this meeting, with particular emphasis on the need for more intelligent testing.

In this context, *operational intelligence* would help in prioritizing areas where anti-doping resources are needed to provide a more proactive response to short-circuit potential or emerging trends or to address existing problems with innovative actions. Intelligence gathering would serve to feed risk assessment processes to identify these specific areas, to evaluate the seriousness of the threat or the actual problem and to deploy resources with the optimal cost-benefit ratio. The question of prioritization is particularly relevant for governments which are facing a question of priority compared to crucial issues, such as healthcare, education, employment or criminality but also in very common instances water and food supply, poverty or human rights for example. When it comes to allowing financial and human resources, one cannot expect countries with such persistent, and sometimes urgent, problems to dedicate resources equivalent to wealthier countries and to support equally what appears to be a minor concern. Although it may be argued that an efficient anti-doping policy may promote and safeguard good health for the active youth of a nation.

Similarly, the *strategic intelligence* process might result in the inclusion of a substance or method to the List or the Monitoring Program, the adjustment of existing law policies, the development of a preventive or educational program, or, conversely, the decision not to legislate or to cancel the current regulation as the problem under scrutiny may be considered insignificant.

In addition, an intelligent approach would allow assessing the efficiency of anti-doping programs by monitoring the geographical and temporal evolution of a phenomenon.

The following chapter proposes to illustrate and test the phenomenon detection and monitoring part of the model by addressing the perceived problem of smokeless tobacco use in sport, in particular snus. The concept of Anti-Doping Intelligence being a wide area of research, focus will be put on this specific function as a first step towards the practical validation and implementation of the theoretical model.

CHAPTER III | SMOKELESS TOBACCO IN SPORT

III.1. Smoke and smokeless tobacco

III.1.1. Consumption forms

A wide variety of consumption patterns exist, from tobacco smoking, in the form of cigarettes, cigars or pipes, to smokeless tobacco (ST) products such as snus, moist and dry snuff and chewing tobacco. Actually, snus is a Swedish type of moist snuff, where non-fermented ground tobacco is contained in tea bags-like pouches that consumers place between the upper lip and gums (Figure 9).

Figure 9. Snus box and use instructions.

Nicotine replacement therapies also contain this natural compound, as marketed in transdermal patches, nasal sprays, inhalers and gums.

Depending on the type of product, concentrations differ to a reasonable extent. On average, a similar content of nicotine is found in cigarette and oral snuff, whereas cigar and chewing tobacco contain only about half of this concentration (62). Accordingly, levels of nicotine intake and metabolism pathways vary along these different trends of tobacco consumption. When smoked and inhaled, nicotine is rapidly absorbed in the lungs, reaching the brain via the bloodstream within 20 s (62). On the other hand, there is little to large buccal absorption depending on the pH, which is directly related to the type of product (63, 64). Chewing tobacco and snus are buffered to facilitate absorption of nicotine through the oral mucosa under its unionized form. A portion of nicotine is usually swallowed with saliva and well absorbed in the small intestine. Actually, the intake of nicotine from moist snuff (3.6 mg) has been estimated to reach approximately twice the absorbed dose from smoking (1.8 mg) (65). Concentration in plasma rises at a slower rate than with smoking and levels are declining over a longer period of time (66) (Figure 10).

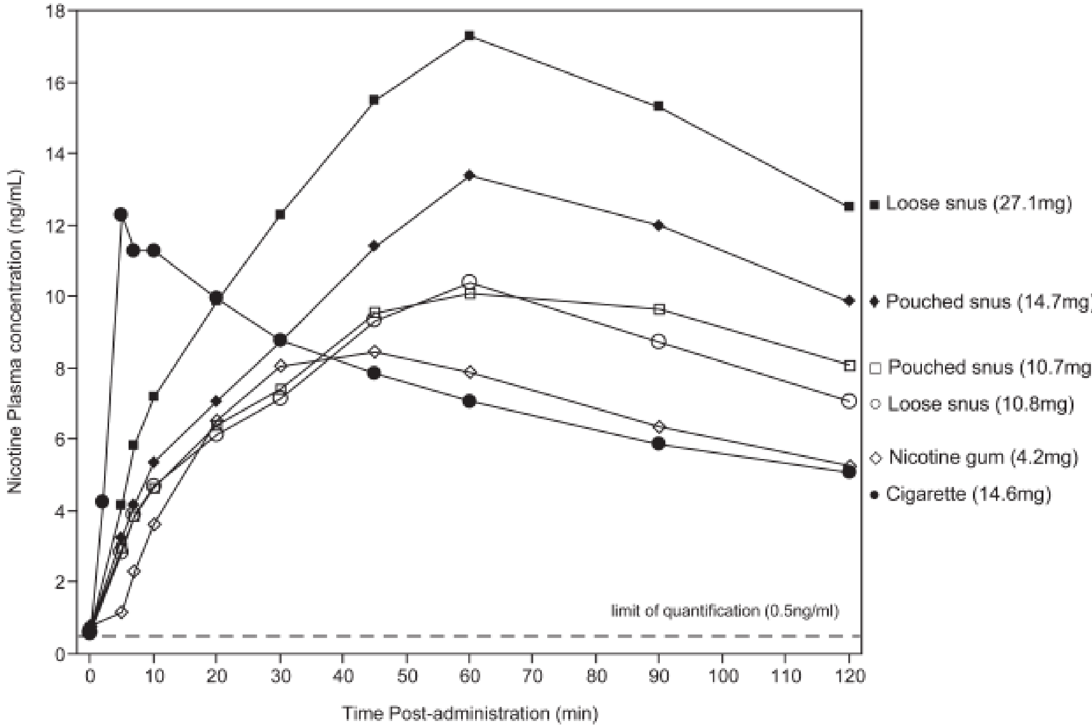


Figure 10. Mean plasma nicotine concentrations at each time point following single use of different tobacco products and nicotine gum. Products (nicotine content): • Cigarette (14.6 mg); □ Pouched snus (10.7 mg); ○ Loose snus (10.8 mg); ♦ Pouched snus (14.7 mg); ■ Loose snus (27.1 mg); ◊ Nicotine gum (4.2 mg). The dashed line represents the limit of quantification (0.5 ng/mL). Reprinted from (66).

To the best of the author's knowledge, controlled studies comparing nicotine urinary profiles after consumption of smoke and smokeless tobacco products are lacking.

On a side note, nicotine is also well absorbed through the skin which is the basis for transdermal delivery that occurs over a long period of time (67).

III.1.2. Tobacco use and health

According to the report on smoking prevalence in World Health Organization (WHO) Member states, adult daily smoking (male and female) in 2006 ranged between 3-19% in Africa, 4-34% in the Americas, 15-29% in South-East Asia, 11-44% in Europe (22% in Switzerland), 6-36% in the Eastern Mediterranean region and 10-47% in the Western Pacific region (68). Noteworthy, significantly higher numbers are found when considering the adult male population only. As an example, a recent survey indicated that 53% of adult Chinese men are daily smokers, while only 2% of the adult women currently use smoke tobacco products, resulting in an average prevalence of 28% for the entire adult population (69).

In particular, smoking has been on the decline in Sweden over the last two decades, making it the only industrialized country to reach the WHO goal of less than 20% adult smokers (70, 71). Nevertheless, ST use has been increasing considerably meanwhile across Scandinavia where snus

consumption is traditional. Actually, a prevalence of snus use of 24% in Swedish men and 3% in women has been reported in 2010 (70, 72). In comparison, a prevalence of 6.4% among young adults between 18-25 and 3.1% among adults over 26 has been reported in the 2010 United States National Survey on Drug Use and Health, pairing with the increase of youth ST use in recent years (73).

Despite public knowledge on toxicity and carcinogenic properties of tobacco smoke components, worldwide consumption is responsible for 5 millions of deaths each year, a number expected to grow up to 8.3 million by 2030 (68). Noteworthy, tobacco use is the main reason behind the escalating burden of disease from the four leading causes of death, including ischaemic heart disease, cerebrovascular disease, infections of the lower respiratory system and chronic obstructive pulmonary disease (74, 75). In addition, trachea, bronchus and lung cancers mortality is an additional major health issue significantly correlated with tobacco consumption. Indeed, due to extremely addictive properties, nicotine plays a major role in the repeated exposure to smoke and consequently to a variety of carcinogens, among which tar, polycyclic aromatic hydrocarbons (PAH), tobacco-specific nitrosamines (TSNA) and pyrolysis products (76-78).

The treatment of nicotine addiction is a global concern which is considered from different perspectives, either supporting smoke cessation programs or preventive and harm-reducing oriented smoke regulation policies. Indeed, current strategies focus on different aspects of the problem, supporting various approaches, from simple medical advising to pharmacotherapy, or from tax increases to implementation of smoke-free environments and distribution of educational material on tobacco consumption (68, 79). Harm reduction is a concept based on the assumption that the incidence of tobacco-related diseases may be reduced by migrating to less harmful tobacco products (80-82). At present, this strategy is best illustrated by a prohibition of smoking in public places growing in popularity worldwide, proving efficient in the intent of reducing both direct and passive exposure while encouraging smokers to quit. As a consequence, the tobacco industry is finding increasing interest to market a diversified range of products to bypass public smoke bans and minimize the health risks concerns, in order to maintain tobacco use among existing smokers and to recruit new tobacco users (65, 82-87). Accordingly, a solid attempt to increase the attractiveness of ST products, in particular snus, is observed throughout Europe and North America (88). However, equivalent addictive properties have been observed when comparing smoked and smokeless nicotine, which is a serious promoting factor for persistent addiction and for smoking initiation (89, 90). In addition, despite avoiding respiratory health issues associated to tobacco smoke, consumption of ST may be responsible for oral, esophageal and, to a less significant extent, pancreatic cancers due to the presence of over 28 carcinogenic constituents, but also heart diseases and serious lesions of the oral tissues (91-96).

III.2. Nicotine

III.2.1. Pharmacology

Nicotine, or 3-(1-methyl-2-pyrrolidinyl)pyridine, is the principal alkaloid found in tobacco leaves, where it acts as a natural insecticide. This molecule exhibits a variety of pharmacological properties sought-after by consumers and responsible for persistent addiction issues. Among the numerous neurotransmitters released in the central nervous system by stimulation of nicotinic cholinergic receptors, dopamine is associated with rewarding experiences (97). Promotion of related positive reinforcing effects results in vigilance and cognitive function enhancement together with relaxation, reduced stress, mood modulation and lower body weight (64, 98). Interestingly, nicotine also triggers a significant increase of pulse rate, blood pressure, blood sugar and epinephrine release owing to simultaneous stimulant and relaxant properties (67, 99). As a consequence, nicotine addiction develops through repeated exposure to experience positive reinforcing effects with relief of withdrawal symptoms (97).

III.2.2. Metabolism

Nicotine is primarily and extensively metabolized in the liver by cytochrome P-450 (CYP) 2A6/aldehyde oxidase-mediated enzymatic conversion to cotinine (Figure 11) (62, 63, 100, 101). Cotinine is further converted to *trans*-3-hydroxycotinine by CYP2A6-mediated oxidation (102). Subsequently, nicotine and cotinine are metabolized into their *N*-glucuronide conjugates by uridine 5'-diphosphoglucuronosyltransferase (UGT) 2B10 and UGT1A4, while *trans*-3-hydroxycotinine is transformed into its *O*-glucuronide conjugate by UGT2B7 (62, 103). Noteworthy, significant inter-individual differences in the glucuronidation of nicotine and metabolites have been observed. Flavin monooxygenase-3 (FMO-3) also converts nicotine into nicotine-*N'*-oxide and cotinine into cotinine-*N*-oxide which are minor metabolites (104).

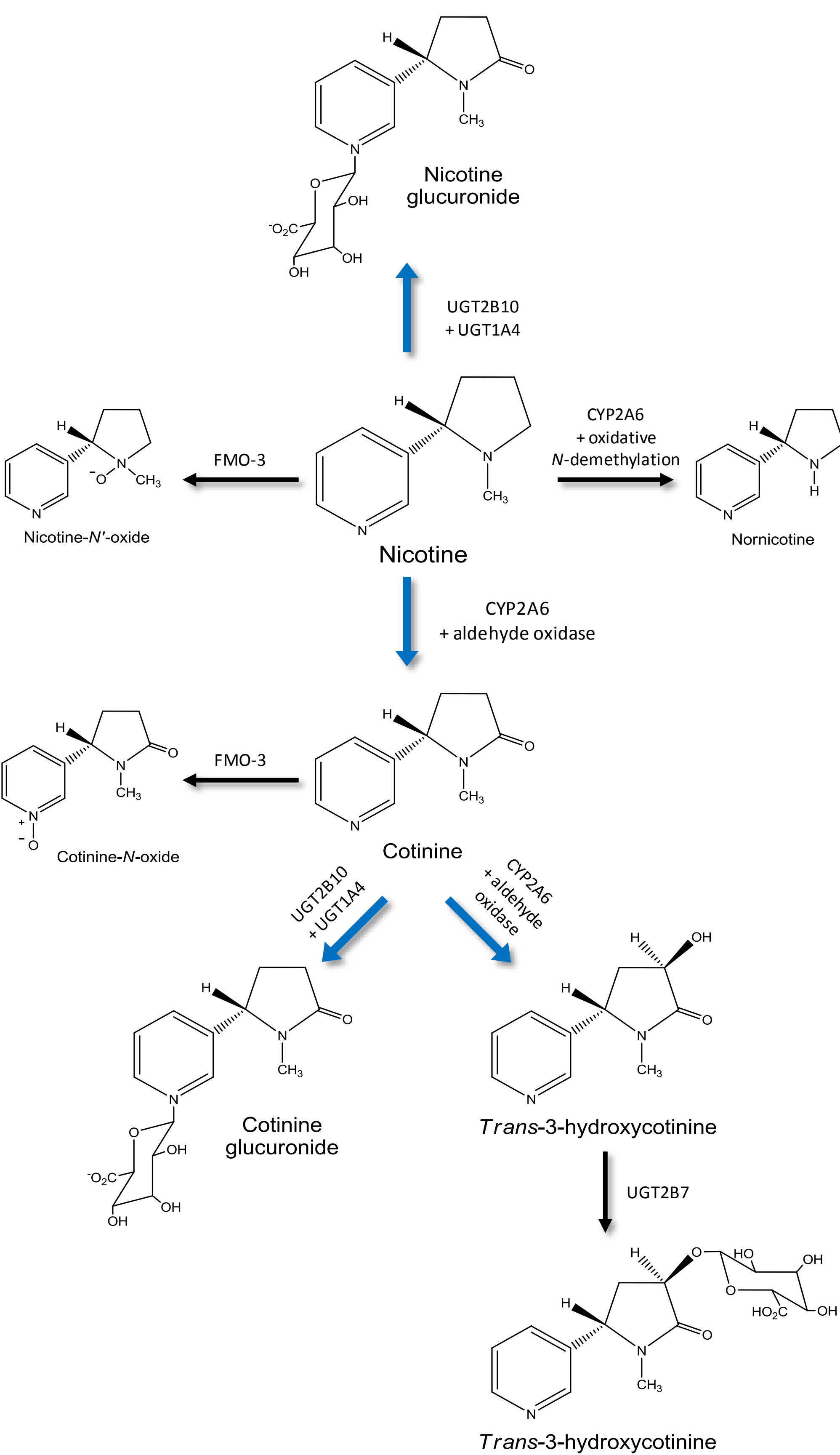


Figure 11. Principal pathways of nicotine metabolism (62, 101).

Quantitatively, the simultaneous determination of free urinary nicotine and related metabolites has been reported in several studies, allowing estimating the share of each metabolic pathway (Table 1).

Molecule	Estimated urinary excretion (% of total nicotine dose)
nicotine	8-10%
nicotine-glucuronide	3-5%
cotinine	10-15%
cotinine-glucuronide	12-17%
trans-3-hydroxycotinine	33-40%
trans-3-hydroxycotinine-glucuronide	7-9%
nicotine-*N′*-oxide	4-7%
cotinine-*N*-oxide	2-5%

Table 1. Summary of the quantitative estimates accounting for nicotine metabolism (62, 100, 105).

Therefore, due to the relatively short half-life of nicotine in urine (about 2 h), investigating nicotine metabolites which exhibit a longer half-life is a prerequisite to provide relevant information on tobacco consumption.

While nicotine accounts for approximately 85-95% of the alkaloids content of tobacco leaves, anatabine, nornicotine and anabasine are, with respect to this order, the most abundant minor alkaloids and excreted in urine unchanged (Figure 12) (62, 93).

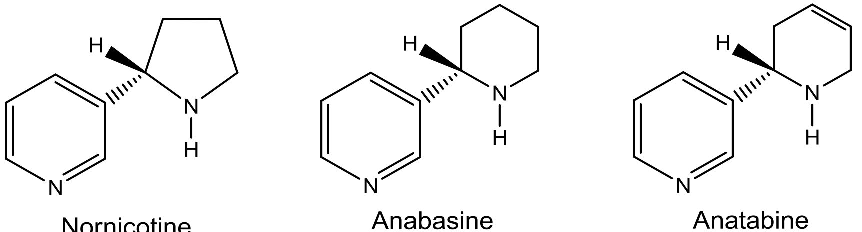


Figure 12. Chemical structure of minor tobacco alkaloids.

Noteworthy, ~60% of urinary nornicotine come from the metabolism of nicotine, while less than 40% comes from tobacco (62).

III.2.3. Smokeless tobacco in sport

A. History of consumption in the United States

On a historical note, smoking and chewing tobacco among the American Major League Baseball (MLB) and Minor League Baseball (MiLB) players dramatically declined during the 1980s, while the use of oral snuff rose sharply (106). Indeed, in the late 1980s and throughout the 1990s, investigators have reported a prevalence varying between 33% and 45% for MLB players and between 45% and 55% for National College Athletic Association (NCAA) baseball players (107, 108). In parallel, ~15% of

high school baseball players used smokeless tobacco in 1995 (109). These figure and the ~31% prevalence among MLB rookie players for the 1999 season indicated that initiation of ST use started before joining the professional ranks for most athletes, whether during their college years or in minor leagues (108). At the same time, the median age at initiation among MLB and MiLB players was ~18 years, with a ~5 years median duration of use (110). Nevertheless, ST consumption is not just restricted to baseball, as highlighted in the 2001 NCCA statistics. Indeed, a prevalence of smokeless tobacco use reaching ~41% in baseball, ~39% in wrestling, ~35% in ice hockey, ~32% in lacrosse, ~29% in football, ~27% in golf, ~25% in water polo, ~20% in soccer, ~17% in track and field, ~13% in tennis and ~12% in basketball was reported (111).

Interestingly, this consumption trend had been following a strong association between athletic performances and use of ST, as illustrated by the marketing strategy of the tobacco industry during this period of time (112). Indeed, rookie and student baseball players have been found to perceive the use of ST and the launch of a successful career as intrinsic, regardless of health risks (108). From a cultural point of view, baseball players have traditionally been serving as role models in the eyes of the youth in America (109). Likewise, the social environment is another significant source of influence, as the majority of high school and college men and athletes have been citing friends' consumption of oral snuff as the primary reason for initiation (113, 114). As a consequence to this profound tendency of the youth to mimic the behavior of peer groups or sport idols, millions of adolescents across the United States have been taking on a consumption habit responsible for persistent nicotine addiction issues and a variety of cancers, heart and mouth diseases (106).

As a response to the high health risk profile associated with smokeless tobacco, the MLB enacted in 1991, and subsequently the NCAA in 1994, a ban on consumption of ST during practices, games and team travels for all MiLB, and college players (108, 111). In a joint effort to help both professional and college players to reduce and/or quit ST use, a cessation guide was developed by the MLB, the Professional Baseball Athletic Trainers Society (PBATS) and the National Cancer Institute (NCI) in 1991 (106). This guide was distributed throughout minor and major leagues as well as in junior colleges and colleges the following year.

In taking this action, the baseball world passed on a strong message to the young generations of athletes on the potential harm of ST products as well as raising consciousness within society on this particular health concern. This is particularly true considering that since the 1970s the tobacco industry had been manipulating free nicotine contained in oral snuff products to promote gradation of dependence, while deliberately tailoring its marketing strategies to target the youth (65, 88, 115). Subsequently, regulation on advertising practices was established in 1998 with the Smokeless Tobacco Master Settlement Agreement (STMSA) which stated the "Prohibition on Youth Targeting" in both direct and indirect advertising, promotion or marketing of tobacco products (88, 116, 117).

Interestingly, this agreement included a prohibition of tobacco brand name sponsorships in any football, basketball, baseball, soccer and hockey league. Since that date, young adults have become an increasingly important sales target to the point of having the highest rate of dual use of smoke and ST compared to adults (118).

B. Doping with smokeless tobacco? Evaluation through Anti-Doping Intelligence

ST consumption first caught the interest of LAD after hearing testimonies from athletes and/or winter sports' teams at an increasing frequency. According to previous researches, nicotine consumption was a popular trend in baseball in the late 80s until the early 2000s, regardless of the level of competition, while the almost non-existent information available on other disciplines depicted it as a common practice in winter sports (106, 119-121). In particular, recent observations within the sport's community pointed at ice hockey. Considering history and consumption patterns of ST throughout the world, North America and Scandinavia are traditional and leading markets for the use of moist tobacco pouches, known as snus (70-72). With a strong ice hockey culture, these geographical regions appear as a natural and fertile ground for the development of snus consumption in this sport. In parallel, recent epidemiological studies inform on the growing popularity of ST in society, which is corroborated by commercial facts and figures of the tobacco business (114, 115, 122-124).

Physiological and pharmacological studies on nicotine indicate a potential for performance-enhancement as a result of stimulant and relaxant properties, as well as almost eliminating adverse effects on the respiratory tract with ST as opposed to tobacco smoke (125-128). Indeed, nicotine has been vastly reported to improve alerting attention in tasks of vigilance, co-ordination, fine motor abilities, memory and cognitive performance, in particular at small to intermediate levels of nicotinic stimulation (125, 127, 129-132). Cognition being an important factor in sport, such optimization of neurobiological function might benefit a variety of sports including sport games or track and field (133, 134). The increased pain tolerance experienced after nicotine intake might also be an interesting advantage (125). Likewise, an improvement of 17±7% in delay to exhaustion due to moderate-intensity exercise has been shown after nicotine administration (129). Similarly to caffeine, nicotine seems to delay central fatigue as impaired central drive is an important factor contributing to fatigue during exercise (133). Nonetheless, there is a debate around this topic as surveys during the 1990s did not point out performance-enhancement properties of nicotine for sport practice (109). However, these assumptions were generally based on performance statistics of athletes rather than controlled studies after nicotine administration.

Nicotine did not appear on the WADA Prohibited List and Monitoring Program when this research was initiated in 2009 and still does not appear on the current List despite satisfying all three

inclusion criteria (4, 5). Indeed, this substance may enhance sport performance in the first place. While health consequences are mainly related to the carcinogenic compounds contained in tobacco or/and coming from the combustion process, nicotine triggers strong addiction mechanisms responsible for repetitive exposure to these harmful chemicals. Therefore, the highly addictive properties of nicotine represent a serious health threat for the athlete. Since the risk assessment by public authorities does not lead to making tobacco or nicotine illegal in countries around the world, WADA raised the concern that the second criterion may not be applicable to ban tobacco in sport. However, this argument is not really relevant as the majority of banned substances are therapeutic agents which are not illegal in society, but at most under controlled medical use or available in pharmacies on presentation of a prescription. Eventually, the negative image associated with tobacco consumption may also alter the spirit of sport.

When collating this information and considering the absence of any regulation on nicotine use in sport, raising interest on the consumption of ST in sport is legitimate for what appears to be an attractive drug from a doping perspective (Figure 13).

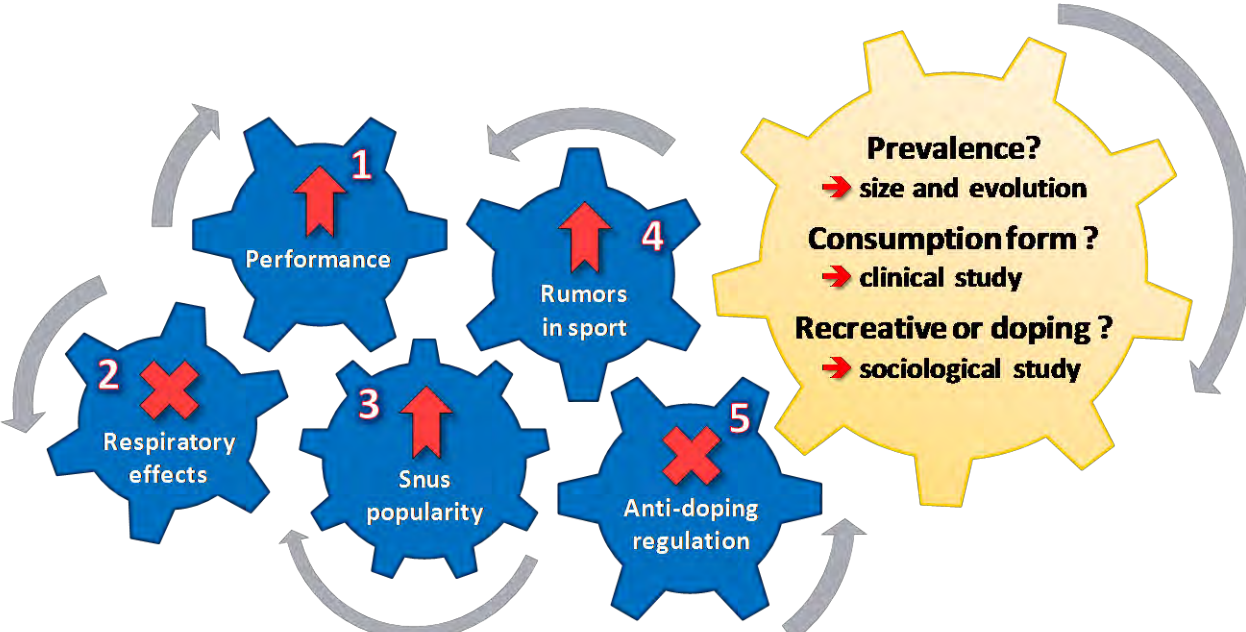


Figure 13. Reasoning mechanism on the interest towards potential doping with ST and related questions relevant to Anti-Doping authorities for decision-making on this topic.

Nonetheless, large-scale comprehensive surveys on nicotine consumption in sport are lacking and to ascertain the frontier between recreational use of a social drug and doping purpose is difficult. Therefore, the following studies propose to illustrate the use of Forensic Intelligence to address social drugs in sport, and more specifically nicotine consumption through ST.

Acquisition of fresh information was essential to feed the logical process to obtain a more complete picture of the phenomenon. As a first step, developing and validating analytical chemistry

tools was a necessary prerequisite to be able to highlight and measure nicotine consumption by the athletes. Further on, prevalence studies could be conducted to collect epidemiological data. These observations were structured and collated with the information carried by testimonies, previous prevalence studies and pharmacological properties of nicotine already integrated into the memory. Afterwards, since sociological data were required to better understand ST consumption mechanisms, a behavioral study based on the randomized-response survey model was designed. In summary, the general idea behind ST studies was to detect, measure, follow and gain knowledge on this phenomenon following an intelligence approach and, ideally, to support anti-doping authorities in decision-making on tobacco consumption in sport.

III.3. Specific projects on smokeless tobacco in sport

During the first step in the elaboration of an intelligence-driven approach for the detection of phenomenon, a prevalence study was conducted during the 2009 Ice Hockey World Championships held in Switzerland (original paper in Appendix V.2.7). The idea to qualify and quantify nicotine consumption in ice hockey was initiated by recurrent testimonies from athletes, team coaches and sports doctors on snus consumption paired along the significant prevalence of snus use in countries with a strong ice hockey culture, such as Scandinavia for instance.

Then, as the phenomenon appeared to be worth further investigations due to the alarming statistics highlighted during this competition, a second prevalence study was conducted to inform on nicotine consumption throughout 2010 across all sports disciplines (original paper in Appendix V.2.6). Accordingly, every IC doping test performed on Swiss athletes or athletes taking part to a sports event in Switzerland was analyzed to provide a picture of the phenomenon as a whole and in each different discipline.

Afterwards, as the prevalence appeared to be significant in numerous of sports, a clinical study was set up to determine nicotine metabolism after smoke or ST administration (protocol in Appendix V.3). The purpose of this project was to develop analytical tools and statistical models based on the metabolic profile of nicotine to distinguish between these different forms of tobacco use. As a result, more precise statistics on ST consumption could be gathered in future monitoring surveys in order to inform on the size and evolution of snus use rather than nicotine consumption regardless of the form of exposure. Likewise, a metabolic-based model could allow assessing specific cases of interest.

Eventually, a sociological study was designed to investigate on the purpose behind snus consumption in ice hockey and its perception by athletes, whether from performance-enhancement or recreational perspectives. Accordingly, every ice hockey player engaged in a Swiss National or

Regional League and every junior player will be asked to fill in a questionnaire addressing nicotine consumption.

III.3.1. Prevalence of nicotine during the '09 Ice Hockey World Championships

A. Introduction

During the first step in the elaboration of an intelligence-driven approach for the detection of phenomenon, a prevalence study was conducted during the 2009 Ice Hockey World Championships held in Switzerland to qualify and quantify nicotine consumption in top-level ice hockey.

A sample preparation protocol and an analytical method for the identification and quantification of nicotine and unconjugated metabolites in urine samples (cotinine, *trans*-3-hydroxicotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) were developed and validated in-house.

The results of this research have been published and the original paper may be found in Appendix V.2.7 (135).

B. Experimental

The analytical method for the simultaneous determination and quantification of nicotine and its four main unconjugated metabolites in urine involved liquid-liquid extraction (LLE) followed by LC-MS/MS analysis in Hydrophilic Interaction Chromatography (HILIC) mode. Apart from a recent publication on nicotine, cotinine and *trans*-3-hydroxycotinine analysis, HILIC columns had never been previously used for such purpose in real biological samples, in particular when including nicotine-*N'*-oxide and cotinine-*N*-oxide (136). Nevertheless, this methodology is primarily dedicated to the analysis of polar compounds, such as basic molecules and related metabolites excreted in urine (137, 138). Owing to the nature of screening procedures for doping agents, a rapid and simple extraction procedure was favored for this prevalence study.

B.1. Sample preparation

Urine samples clean-up is based on a method previously published and adapted to our particular needs and matrix (139). An aliquot of urine (1 mL) was spiked with 10 μL of 10 μg/mL deuterated internal standard (IS) solution (*d4*-nicotine, *d3*-cotinine and *d3*-*trans*-3-hydroxycotinine) and diluted with 1 mL phosphate buffer (0.2 M, pH 7.0) prior to vortex mixing. LLE was performed with 2.5 mL chloroform: propan-2-ol (95:5, v/v) for 10 min using a rotator unit. After centrifugation for 5 min at 2500 rpm, the organic layer was evaporated to dryness under a gentle air stream at 50 °C and

reconstituted in 1 mL ACN: ammonium formate (10mM, pH 3.0) (98:2, v/v) prior to LC-MS/MS injection.

B.2. LC conditions

Separation was carried out on a LC-MS/MS system using a Rheos 2000 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and an HTS Pal autosampler (CTC analytics AG, Zwingen, Switzerland). Hydrophilic Interaction Chromatography was performed on a Phenomenex Luna® HILIC column (150 mm × 3.0 mm, 5 μm) (Brechbühler AG, Schlieren, Switzerland) with a guard column SecurityGuard™ HILIC (4 x 2.0 mm) (Brechbühler AG, Schlieren, Switzerland) added to the analytical column. The column temperature and the autosampler tray were set at 30 °C and 4 °C, respectively. Mobile phase consisted of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 0.3 mL/min, while partial loop injection volume was 10 μL with a 20μL loop. The initial mobile phase condition was 98% A for 3 min, which was decreased linearly to 35% and held from 10 to 13 min, then increased back to 98% to re-equilibrate the column from 13.1 to 16 min.

B.3. Linear Trap Quadrupole-MS parameters

Analyses were performed using a linear ion trap mass spectrometer LTQ-MS (ThermoFinnigan, San Jose, CA, USA) equipped with an atmospheric pressure ionization (API) interface, Ion MAX™, operated in positive ESI mode. MS operating conditions were set as follows: spray voltage = 5.0 kV; heated capillary voltage and temperature of 10 V and 320 °C, respectively; isolation width of 1.5 Da; activation time = 30 ms; activation q of 0.250 and scan time was fixed at 30 ms. Sheath gas, auxiliary gas and sweep gas (nitrogen) were set at 20, 5 and 1.5, respectively.

B.4. Identification criteria

Identification criteria were defined according to the WADA Technical Document addressing qualitative assays (140). The retention time (t_R) tolerance window corresponding to the analyte and the quality control (QC) of the same batch must be within the range of ± 2%. Also, three diagnostic ions are required, which may include the precursor ion, with an intensity ≥ 5% of the most intense diagnostic ion of the MS/MS spectrum. Eventually, a Signal-to-Noise ratio ≥ 3 must be be observed, with a relative intensity of any of the ions not differing by more than 10% (absolute) or 25% (relative) from the QC urine.

B.5. Method validation

Calibration curves

Experiments were conducted following the guidelines on bioanalytical method validation from the US Food and Drug Administration (FDA) and the recommendation of the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 (141, 142).

A pool of six urine samples from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days was prepared to obtain negative urine (Uneg) for the validation process.

Also, according to the pharmacological effects of nicotine and keeping in mind a doping perspective, only recent consumption of nicotine was of relevant interest. Indeed, trace levels in the 1 ng/mL scale would not provide meaningful quantitative information on the consumption behavior. Thus, in order to ensure statistical significance for further discrimination between recreational, environmental or doping use, choosing a concentration of 10 ng/mL for the lowest calibrator through the validation procedure looked appropriate to ensure quality quantitative results while maintaining minor bias.

Therefore, the calibration was established over the 10-10'000 ng/mL range for nicotine, cotinine and *trans*-3-hydroxycotinine and 10-5'000 ng/mL range for nicotine-*N'*-oxide and cotinine-*N*-oxide. A set of three validation series was achieved, with calibration standards at six concentration levels (k = 6) and validator standards (QC) at four concentration levels (k = 4) prepared in triplicate (n = 3) each time. Calibration curves were built from the peak area ratio of nicotine and metabolites to *d4*-nicotine for nicotine, *d3*-cotinine for cotinine and *d3-trans*-3-hydroxycotinine for *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide. Accuracy was defined as the closeness of agreement between the theoretical and the average measured concentrations. Precision expressed the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Repeatability and intermediate precision are two of the three different levels of precision. Repeatability was expressed as the relative standard deviation (RSD) of the ratio of the intra-day standard deviation and the theoretical value at each concentration level (143). Intermediate precision was expressed as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration level. An accuracy profile was built for each analyte, combining accuracy and intermediate fidelity variance in the dosing range (144, 145). Data were processed and reported with Xcalibur LCquan package software from ThermoFinnigan and calculation were performed on Excel 2007 from Microsoft.

The lower limit of quantification (LLOQ) was determined as the lowest QC sample with an acceptable accuracy, repeatability and intermediate precision fitting for purpose. Quantitative analysis of nicotine and metabolites in real urine samples was performed using a three-points

calibration curve determined and fitted by a linear least-squares regression of the peak area ratios between the analyte and the IS versus concentrations. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level of a blank urine preparation.

Selectivity

Influence of endogenous matrix compounds was determined by analyzing urine samples from 6 individuals certified as negative (< LOD) for nicotine and metabolites. Each sample was extracted in triplicate to highlight the presence of potential interfering matrix compounds within selected tolerance windows.

Accordingly, influence of exogenous xenobiotics was determined by analyzing urine samples from over 250 individuals with different nicotine consumption habits who reported the use of very various substances appearing on the 2009 Prohibited List and Monitoring Program.

Carry-over

Carry-over was evaluated correspondingly by injecting a blank urine sample subsequently to the analysis of the highest calibrator. This experiment was conducted in triplicate.

Matrix effects

Matrix effects on the ionization response and extraction efficiency were further evaluated along the recommendations published elsewhere (146). A neat solution was fortified at low, medium and high concentration in the initial mobile phase ACN: ammonium formate 10 mM (pH 3.0) buffer (98:2) (a), while a set of 6 negative urines was also fortified in duplicate prior to extraction (b) and another set of blank urine specimens was extracted and fortified only after (c). By comparing the absolute peak areas of two sets of solutions, matrix effect and extraction efficiency can be evaluated, as reported below (Eqs. (1) - (2)).

$$\text{Matrix effect (ME)} = c/a \qquad (1)$$

$$\text{Extraction efficiency (RE)} = b/c \qquad (2)$$

Stability

The effect of storage conditions was studied by performing a longitudinal stability assay. Analyte stability was evaluated by monitoring the influence of successive freeze and thaw cycles of QC urine samples at low, medium and high concentrations over a period of six months. As real urine samples were stored at -20 °C in a sealed box since their collection, the QCs were handled likewise and

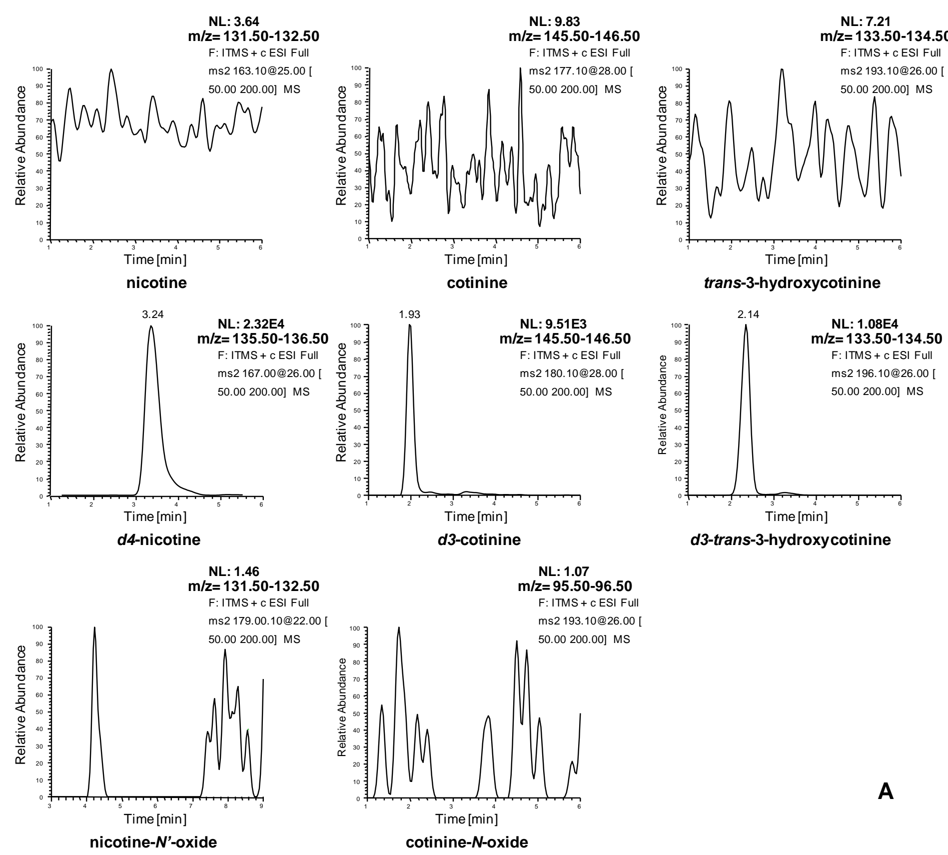
defrosted at ambient temperature twice a month for LC-MS/MS analysis. The initial integrated peak area was defined as 100%.

C. Results and Discussion

C.1. Method development

LC-MS/MS analyses

A complete separation of nicotine and metabolites in urine specimens was achieved by hydrophilic interaction chromatography using a gradient of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 0.3 mL/min (Figure 14). Indeed, HILIC mode allowed successfully isolating each analyte by providing adequate retention of polar compounds and excellent peak shape. Sensitivity was also optimized since using a mobile phase highly enriched in polar organic solvent ensures an efficient ionization towards the molecules of interest (137). Likewise, reduced endogenous matrix interferences resulted in very clean chromatograms and a high throughput was obtained due to the feasibility of using a higher flow rate.



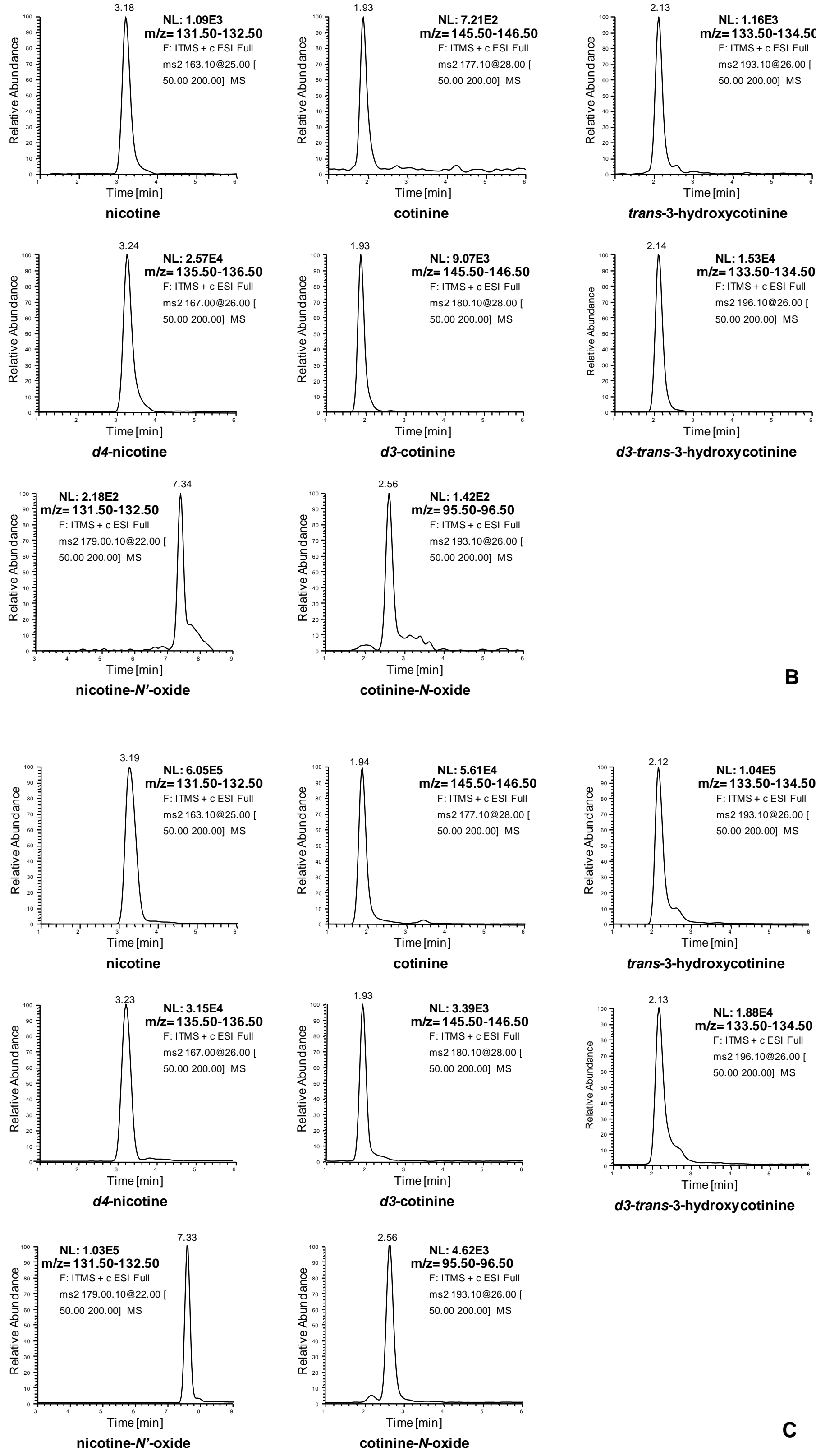


Figure 14. LC-MS/MS chromatograms of a blank urine (A) compared to a urine specimen containing nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide at a concentration of 10 ng/mL (B), both with IS spiked at 100 ng/mL. A chromatogram of a urine sample from a hockey player containing the above mentioned metabolites at 1'074, 1'415, 3'739, 2'586 and 459 ng/mL, respectively, is also depicted (C). Quantification ion transitions are in bold.

Repeatability of the retention times (t_R) was evaluated by calculating mean values variability over the set of three validation series which consisted in 45 extracted samples (Table 2). The RSD obtained were found satisfactory for all the compounds of interest, ranging from 1.8 - 4.1%.

Molecule	SRM transition (m/z)*	Collision energy (eV)	t_R (min)
nicotine	163 → **132**, 120, 106	25	3.18
d4-nicotine	167 → **136**, 124, 110	26	3.24
cotinine	177 → **146**, 98, 80	28	1.93
d3-cotinine	180 → **146**, 101, 81	28	1.93
trans-3-hydroxycotinine	193 → **134**, 118, 80	26	2.13
d3-trans-3-hydroxycotinine	196 → **134**, 89, 80	26	2.14
nicotine-*N'*-oxide	179 → **132**, 130, 117	22	7.34
cotinine-*N*-oxide	193 → 134, **96**, 98	26	2.56

*Quantification ion transitions are in bold.

Table 2. SRM parameters and retention times of the analytes.

Direct infusion of individual standard solutions, with a flow rate and mobile phase composition corresponding to the elution time from the LC column, allowed optimization of tandem mass spectrometry parameters. Gas streams, spray voltage, heated capillary voltage and temperature, isolation width and compound specific normalized collision energies were manually tuned, resulting in a high sensitivity fragment spectra with a precursor ion response < 10% in abundance. SRM transitions, collision energies and retention times for each analyte are provided in Table 2.

LLE

Sample preparation in dope testing favors time and cost efficient procedures providing satisfactory matrix clean-up and recovery. Thus, the selective extraction protocol for urine samples used in this work was performed with a single LLE. Nicotine and metabolites were neutralized with phosphate buffer at pH 7.0, triggering the extraction with chloroform: propan-2-ol (95:5, v/v). Extraction was followed by evaporation of the organic phase and reconstitution in the initial mobile phase mixture. This simple, cost and steps-limited methodology provided very clean extracts of urine samples containing nicotine and metabolites. Noteworthy, a batch of 50 items could be prepared within 1 h, allowing a significant workflow of analysis.

RE ranged from 70.4 to 100.4% depending on the analyte, with evidence of good repeatability (RSD < 15%), and showed only slight dependency on the concentration level (Table 3).

Molecule	Concentration (ng/mL)			Recovery (%)			RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
nicotine	10	5’000	10’000	95.2	93.4	89.9	3	2	3
cotinine	10	5’000	10’000	99.8	97.6	95.7	9	5	1
trans-3-hydroxycotinine	10	5’000	10’000	70.4	71.6	73.1	5	3	2
nicotine-*N′*-oxide	10	2’500	5’000	82.3	83.1	83.2	3	1	0
cotinine-*N*-oxide	10	2’500	5’000	76.6	80.5	82.7	0	0	1

Table 3. Recovery and related RSD of nicotine and metabolites at low, medium and high concentrations (n = 5).

Indeed, RE for *trans*-3-hydroxycotinine was below what was obtained for the other metabolites. This may result from the p*Ka* of *trans*-3-hydroxycotinine being much lower compared to the pH of the phosphate buffer.

C.2. Assay validation

Calibration curves

Concentration ranges were initially determined according to expected levels in urine for nicotine and metabolites, while considering both the pharmacological effects of nicotine and a doping perspective which focuses on recent consumption only (147, 148). Thus, in order to ascertain statistical significance for further discrimination between recreational, environmental or doping use, a LLOQ of 10 ng/mL proved to ensure very accurate quantification.

Determination of the best calibration was performed with the evaluation of different curves fitting. Combining accuracy and intermediate fidelity variance allowed building a profile of confidence interval in the dosage range for each target compound (144, 145). According to these accuracy profiles, unweighted linear least-squares regression was found to provide the highest quality results and was chosen for quantification purpose. Due to the linear response, calibration standards were subsequently reduced to LLOQ, medium and ULOQ concentration levels (k = 3) and QCs to low, medium and high concentration levels (k = 3) with accuracy profiles of comparable quality (Figure 15).

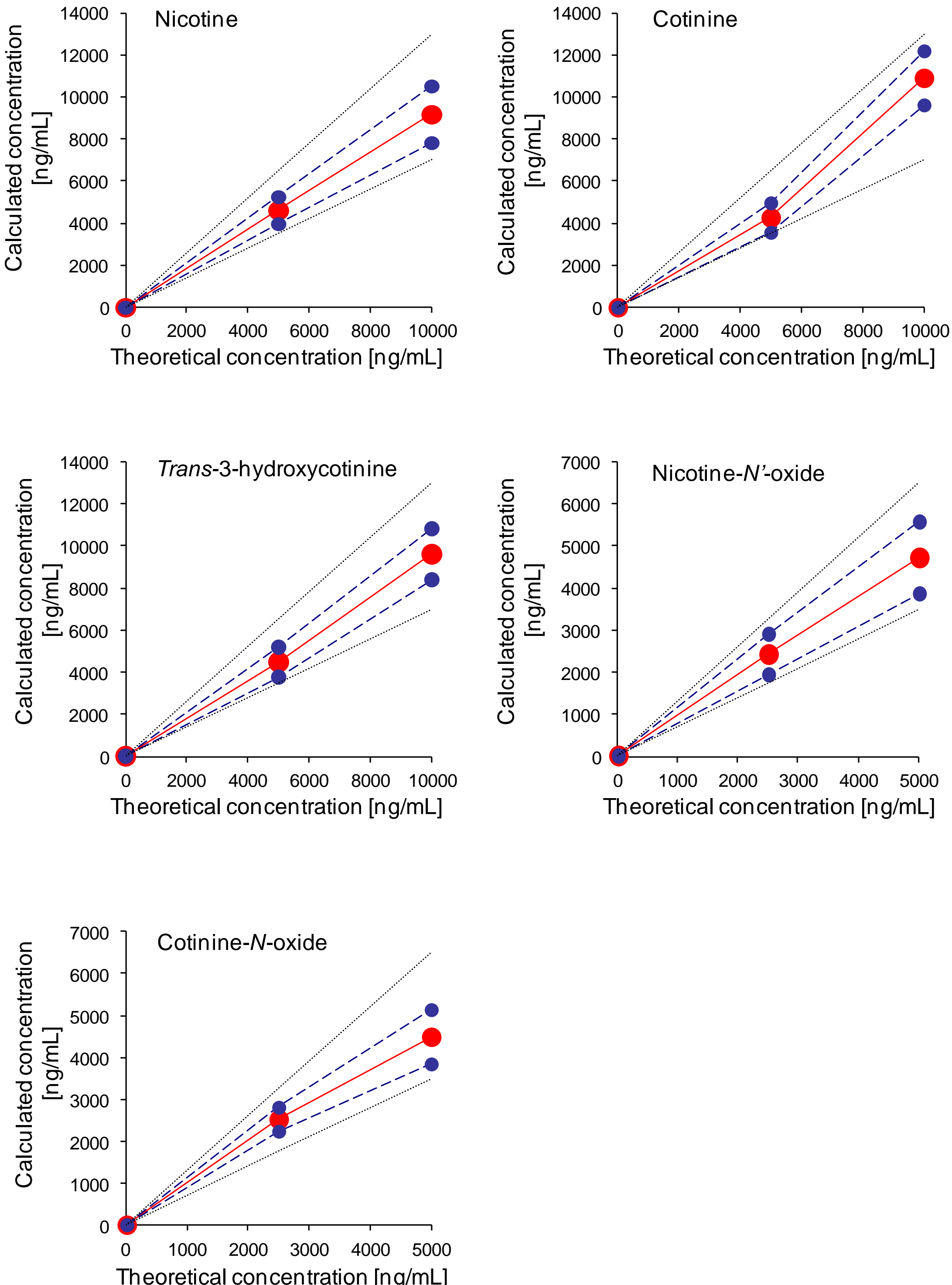


Figure 15. Absolute accuracy profiles for nicotine and metabolites. The solid line indicates the accuracy and the dashed lines represent the accuracy calculated as confidence interval (145). The dotted lines depict the acceptance limits of ± 30 %.

Indeed, accuracy, repeatability and intermediate precision assessments met the guidelines for bioanalytical method validation over the assay range (Table 4). Noteworthy, coefficient of determination (R^2) corresponding to the initial calibration curve for each compound (k = 6) were

greater than 0.95, while R^2 with a reduced number of calibrators (k = 3) were greater than 0.99. This significantly improved the applicability of this method, allowing a better workflow and simplified calibration.

Molecule	Concentration (ng/mL)	Accuracy (%CV)	Precision	
			Repeatability (%CV)	Intermediate precision (%CV)
nicotine	10	98.4	7.6	8.1
	5’000	92.5	6.5	6.6
	10’000	91.8	6.8	6.9
cotinine	10	105.0	6.4	9.2
	5’000	85.5	6.9	7.2
	10’000	109.1	2.7	6.6
trans-3-hydroxycotinine	10	96.6	7.6	7.8
	5’000	89.6	6.0	7.3
	10’000	96.1	5.6	6.2
nicotine-*N’*-oxide	10	102.4	5.5	5.3
	2’500	96.8	9.4	9.9
	5’000	94.3	7.7	8.8
cotinine-*N*-oxide	10	103.9	5.3	5.7
	2’500	101.1	5.4	5.9
	5’000	89.9	4.6	6.7

Table 4. Assay validation parameters for nicotine and metabolites (n = 3).

Therefore, suitability of direct quantification of nicotine and metabolites in urine with this LC–MS/MS method was proven, in particular for nicotine, cotinine and *trans*-3-hydroxycotinine along with nicotine-*N’*-oxide and cotinine-*N*-oxide at concentration ranges of 10 - 10’000 ng/mL and 10 - 5’000 ng/mL, respectively.

Also, the LOD was found to stand around 500 pg/mL for all compounds.

Selectivity

Selectivity tests towards endogenous matrix compounds were conducted on 6 different urine samples obtained from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days. After extraction in triplicate followed by LC-MS/MS analysis, no interfering endogenous molecules were observed within selected scan windows since ion identification criteria, including retention times, ion transitions and ion ratios, were not met (140).

Likewise, assessment of potential influence of exogenous xenobiotics was performed on a set of over 250 urine samples collected from individuals of the general population who reported joint exposure of nicotine and different substances present in the 2009 Prohibited List and Monitoring Program. Noteworthy, influence of stimulants most commonly found in urine of hockey players was

evaluated, among which caffeine and pseudoephedrine. Again, after extraction and LC-MS/MS analysis, no interfering exogenous xenobiotics were observed within selected scan windows according to the criteria mentioned earlier.

Carry-over

Carry-over was evaluated accordingly, after injection of the highest calibrator (10’000 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 5’000 ng/mL for nicotine-*N’*-oxide and cotinine-*N*-oxide), followed by the analysis of a blank urine sample. This procedure was repeated three times successively. None of the target compounds were detected, demonstrating the absence of any carry-over effect.

Matrix effect

ME evaluation by comparison of the signals observed in urine and in the neat solution indicated ion enhancement or suppression depending on the target analyte and concentration. Indeed, nicotine, *d4*-nicotine, nicotine-*N’*-oxide and cotinine-*N*-oxide showed significant ion enhancement at low, medium and high concentrations, while cotinine, *d3*-cotinine, *trans*-3-hydroxycotinine and *d3*-*trans*-3-hydroxycotinine showed substantial ion suppression at low concentration (data not shown). According to the good repeatability of these assessments (RSD < 15%), along with the satisfactory sensitivity and selectivity of the method, ME influence on the results quality was not significant.

Stability

The influence of storage conditions was evaluated by performing a longitudinal stability assay of QC samples every two weeks over a period of six months. Indeed, these samples experienced freeze and thaw cycles involving successive storage at -20 °C in a complete dark environment and defrost at room temperature, corresponding to storage and analysis conditions during this study.

Referring to the limited variation observed in the peak areas (RSD < 15%), the storage conditions described previously ensured a high stability of all analytes over this particular period of time.

D. Application to the Ice Hockey World Championships samples

As part of regular doping control protocols during the 2009 Ice Hockey World Championships held in Switzerland, urine samples were collected shortly after every game on two players of each team (n = 72). After approval by the International Ice Hockey Federation (IIHF) and the Swiss National Anti-Doping Agency (Antidoping Switzerland or ADCH) and as required by the 2009 International Standards for Laboratories (ISL), article 19 of the World Anti-Doping Code and articles 24-27 of the UNESCO Convention against doping in sport, a minimum storage period of three months and

complete removal of identification means were ensured prior to use of these samples for research purpose (3, 6, 149). Noteworthy, storage time did not exceed six months.

Compounds of interest were quantified in duplicate using a three-point calibration curve together with three urine-based QCs, as described previously. Also, a qualitative value was assigned to target analytes metabolites detected in the sub-LLOQ concentration range, namely *traces*. Concentrations distribution for nicotine and metabolites as quantified in urine specimens are illustrated in Figure 16. Nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide concentrations ranged between 11 – 19'750 ng/mL, 13 – 10'475 ng/mL, 10 – 8'217 ng/mL, 11 – 3'396 ng/mL and 13 – 1'640 ng/mL, respectively.

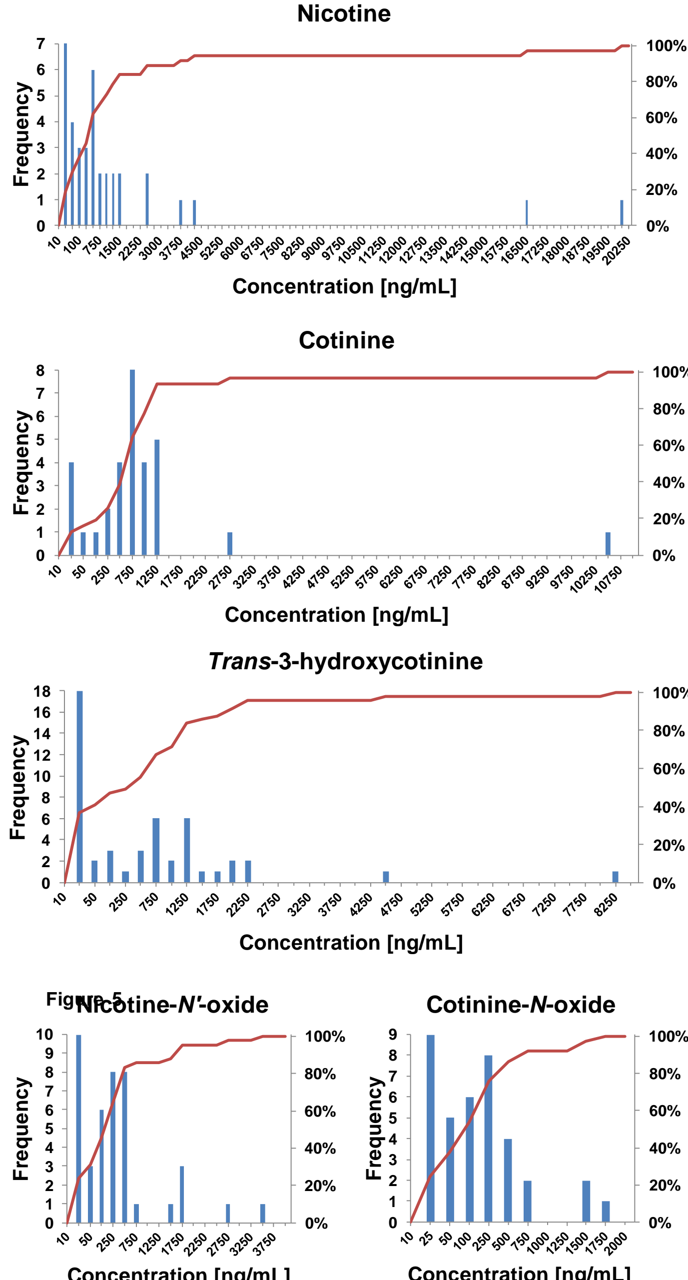


Figure 16. Concentrations distribution for nicotine and metabolites. The solid red line indicates the cumulative percentage.

Traces of nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide were detected in 87%, 91%, 94%, 97% and 97% of samples, respectively (Table 5). Noteworthy, at least one of the three major metabolites was present in every sample. These findings suggest that every athlete was exposed to nicotine, either environmentally or from active consumption, during the competition period. Such results should be carefully interpreted regarding prevalence studies on smoking in society and on environmental tobacco exposure (ETS) among non-smokers. Indeed, smoking prevalence has been reported by WHO as ranging from 15 - 44% depending on the country participating to the 2009 IIHF World Championships (68). These numbers are usually higher for the male population only. Actually, the sixteen participating national teams were Austria, Belarus, Canada, Czech Republic, Denmark, France, Finland, Germany, Hungary, Latvia, Norway, Russia, Slovakia, Sweden, Switzerland and the United States. In 2009-2010, daily snus consumption prevalence among the countries in competition was the highest for Sweden and Norway, concerning 24% and 33% of adult males, respectively (70, 72, 128). Also, ETS for a period of at least one hour per day reached 21% in Switzerland, which hosted the competition (150). However, both facts may explain only parts of such extensive nicotine exposure when considering that athletes are significantly less likely to smoke or to be exposed to ETS than the general population, in particular within the confined environment of such a major competition.

Analyte	Concentration range		
	x ≥ LOD	x ≥ LLOQ	**Active exposure**
nicotine	87.5%	51.4%	36.1%
cotinine	91.7%	43.1%	36.1%
trans-3-hydroxycotinine	94.4%	68.1%	40.2%
Cumulative exposure	**100%**	**83.3%**	**52.7%**
nicotine-*N'*-oxide	97.2%	58.3%	44.4%
cotinine-*N*-oxide	97.2%	51.4%	38.8%

Table 5. Prevalence of IIHF urine samples exposed to nicotine or metabolites depending on the concentration range (n = 72).

Furthermore, above LLOQ levels of the previously mentioned compounds were measured in 51%, 43%, 68%, 58% and 51% of samples, respectively. One of the three major metabolites was detected at such concentrations in 83% of samples. According to the pharmacokinetics of nicotine, exposure may have occurred within the last three days previous to the games for approximately eight ice hockey players out of ten (151).

Prevalence of nicotine consumption, in the form of smoke or smokeless nicotine, close to and/or during the games was evaluated by hypothesizing conservative concentration limits for active

consumption (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine) (151, 152). Also, chances of exposure to serious environmental smoke within the few hours prior to games of such importance were excluded. Accordingly, active nicotine use was highlighted in 36% - 40% of samples, depending on the target compound (Table 5). Noteworthy, at least one of the three major metabolites was present at such levels in 53% of the urine samples, emphasizing a significant prevalence of nicotine consumption amongst ice hockey players close to and/or during the games.

Interestingly, two samples presented highly elevated nicotine concentrations exceeding 10’000 ng/mL. Such acute exposure to nicotine is hardly achievable for a regular consumer (148, 153). Considering the detrimental respiratory effects due to extensive smoking prior to sport practice and as players do not smoke during games, smokeless nicotine use is the most likely hypothesis and a doping purpose may be hypothesized (154-157). This is also supported by the relatively short half life of nicotine. Thus, according to the quantitative measurements, the likelihood of smokeless nicotine use for these two samples is very high. However, due to the lack of clinical studies and statistical models to address the metabolic-based distinction between different forms of nicotine consumption, this hypothesis could not be verified for these two samples or be evaluated for samples with lower concentrations of nicotine and metabolites. Likewise, studies on the relationship between nicotine levels and doping are missing, hence the careful assumptions made here.

In the context of understanding use of smoked tobacco or ST from concentration data, the question of steady-state concentrations in chronic smokers is important. According to the literature, only limited accumulation has been observed for plasma levels of nicotine after multiple doses (158). Despite the lack of consistent urinary data on this topic, a similar situation is very likely to be observed. Likewise, night abstinence allows reducing nicotine down to very low levels, even for chronic users (62, 151, 159). Therefore, the issue of steady-state nicotine concentrations was not considered as a significant bias for the interpretation of the data.

E. Conclusion

In summary, this work highlighted an alarming prevalence of active nicotine consumption of nicotine during ice hockey games at top-level far superior to the prevalence of smoking and ST use in society. As this preliminary study focused on a single event and discipline, regardless of the type of product and the recreational or doping character of nicotine use, a larger-scale study was required to assess the extent of this phenomenon.

III.3.2. Prevalence of nicotine in sports during 2010

A. Introduction

Considering these preliminary results, a one-year monitoring study was conducted to assess the prevalence of nicotine consumption across all sports disciplines tested IC by ADCH over a one-year period of time covering 2010 and 2011.

In order to deal with a very high volume of samples, a straight-forward dilute-and-shoot (DS) sample treatment procedure and a ultra-high pressure liquid chromatography-triple quadrupole mass spectrometry (UHPLC-TQ-MS/MS) method were developed for the identification and quantification of nicotine, its major metabolites (cotinine and *trans*-3-hydroxicotinine), minor metabolites (nicotine-*N'*-oxide and cotinine-*N*-oxide) as well as minor tobacco alkaloids (anabasine, anatabine and nornicotine).

Again, the results of this research have been published and the original paper may be found in Appendix V.2.6 (160).

B. Experimental

The previous analytical method for the simultaneous determination and quantification of nicotine and its four main unconjugated metabolites in urine involved liquid-liquid extraction (LLE) followed by LC-MS/MS analysis in Hydrophilic Interaction Chromatography (HILIC) mode. While this approach was found satisfactory for the analysis of a small number of samples, a more time- and cost-efficient method with comparable, if not superior, sensitivity and selectivity was required for a year-long monitoring of nicotine use. Therefore, a semi-automated DS treatment was developed to replace the LLE procedure and the HILIC method was transposed from the LC-MS/MS system to a UHPLC-TQ-MS/MS instrument.

B.1. Sample preparation

An aliquot of urine (20 μL) was loaded by a Freedom EVO® 150 pipetting robot (Tecan Systems, Männedorf, Switzerland) on a 96-well plate, spiked with 20 μL of deuterated internal standard (IS) solution (*d4*-nicotine and *d4*-anatabine at 250 ng/mL, *d3*-cotinine and *d3*-*trans*-3-hydroxycotinine at 50 ng/mL in ACN) and diluted with 760 μL ACN prior to vortex mixing, corresponding to a 40-fold dilution. After centrifugation for 5 min at 2500 rpm, the supernatant was transferred to another 96-well plate with the pipetting robot and followed by UHPLC-MS/MS injection.

When concentration of a target analyte was determined as superior to the upper limit of quantification (ULOQ), a second aliquot of urine was prepared likewise but spiked with 40 μl of IS

solution and diluted with 1'540 µl of ACN, resulting in an 80-fold dilution. Eventually, measured concentration was multiplied by 2 after UHPLC-MS/MS analysis.

B.2. UHPLC conditions

Separation was carried out on an Acquity UPLC System (Waters, Milford, USA) with a Waters Acquity UPLC BEH HILIC column (2.1 x 50 mm, 1.7 µm) preceded by a Waters Acquity UPLC BEH HILIC VanGuard pre-column (2.1 x 5 mm, 1.7 µm). Column and autosampler tray temperatures were set at 30 °C and 10 °C, respectively. Mobile phase consisted of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 800 µL/min. Initial mobile phase was 99% A held for 0.7 min, decreased linearly to 75% over 1.9 min in a first step and then to 40% over 0.35 min. The column was washed during 0.25 min and mobile phase increased back to 99% to re-equilibrate the system for 1.8 min. Injection volume was fixed at 2 µL in full loop mode.

B.3. Triple Quadrupole-MS parameters

Analyses were performed using a Waters Xevo™ TQ-S triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode. MS operating conditions were set as follows: desolvation gas flow set at 600 L/h at a temperature of 550 °C, capillary voltage at 3.0 kV in positive mode, cone voltage and collision energies optimised for each compound (Table 6). The source temperature was 150 °C, the cone gas flow was set to 150 L/h and the collision gas flow was 0.15 mL/min.

Molecule	MRM transition (m/z)*	Collision energy (eV)	Cone voltage (V)	t_R (min)
nicotine	163 → **132**, 117, 84	15	18	2.04
d4-nicotine	167 → 136, **134**, 121	20	20	2.05
cotinine	177 → **146**, 98, 80	16	15	1.02
d3-cotinine	180 → 146, **101**, 81	20	20	1.04
trans-3-hydroxycotinine	193 → **134**, 86, 80	20	16	0.97
d3-*trans*-3-hydroxycotinine	196 → **134**, 89, 80	20	20	0.99
nicotine-*N'*-oxide	179 → **132**, 130, 117	14	10	2.75
cotinine-*N*-oxide	193 → 134, **96**, 79	18	14	1.90
anabasine	163 → 146, 130, **117**	20	40	2.23
anatabine	161 → 144, 117, **107**	12	26	2.13
d4-anatabine	167 → 148, 132, **111**	20	20	2.13
nornicotine	149 → 132, **117**, 106	20	34	2.27

*Quantification ion transitions are in bold.

Table 6. MRM parameters and retention times of the analytes.

B.4. Identification criteria

Identification criteria were defined according to the WADA Technical Document addressing qualitative assays, as described in the previous study (140).

B.5. Method validation

Calibration curves

Similar to the previous study, a method validation approach was adopted considering guidelines on bioanalytical method validation from the US Food and Drug Administration (FDA) and the recommendations of the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 (141, 142).

A pool of six urine samples from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days was prepared to obtain negative urine (Uneg) for the validation process.

Calibration was established over the 10-10’000 ng/mL range for nicotine, cotinine and *trans*-3-hydroxycotinine and 10-5’000 ng/mL for nicotine-*N′*-oxide, cotinine-*N*-oxide, anabasine, anatabine and nornicotine. A set of three validation series was achieved, with calibration standards at six concentration levels ($k = 6$), with an additional point at ULOQ level after an 80-fold dilution, and validator standards (QC) at four concentration levels ($k = 4$), each being prepared in triplicate ($n = 3$). Calibration curves were built from the peak area ratio of nicotine to d4-nicotine, cotinine to d3-cotinine, *trans*-3-hydroxycotinine, nicotine-*N′*-oxide and cotinine-*N*-oxide to *d3*-*trans*-3-hydroxycotinine, nornicotine, anatabine and anabasine to *d4*-anabasine. Accuracy was defined as the closeness of agreement between the theoretical and the average measured concentrations. Precision expressed the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Repeatability and intermediate precision are two of the three different levels of precision. Repeatability was defined as the RSD of the ratio of the intra-day standard deviation and the theoretical value at each concentration (143). Intermediate precision was expressed as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration. Accuracy profiles were built for each compound of interest, combining accuracy and intermediate fidelity variance in the dosing range (144, 145).

The LLOQ and the ULOQ were determined as the lowest and the highest concentrations of QC samples with an acceptable accuracy, repeatability and intermediate precision. Quantitative analysis of target compounds in real urine specimens was performed using a three-points calibration curve determined and fitted by a linear least-squares regression of the peak area ratio of the analyte and

the IS versus concentrations. The LOD was defined as the concentration that produced a Signal-to-Noise ratio ≥ 3.

Selectivity

Selectivity assessment of interfering endogenous matrix compounds within selected tolerance windows was carried out by analyzing urine specimens certified as negative (< LOD) regarding nicotine and metabolites. For this purpose, a triplicate sample treatment of urine samples from 6 nicotine-abstinent subjects who reported no exposure to environmental smoke within the last 5 days was conducted.

Accordingly, influence of exogenous xenobiotics was considered when a substance appearing on the 2010 Prohibited List and Monitoring Program was highlighted by routine sample screening procedures.

Carry-over

Injection of a blank urine sample after analysis of the highest calibrator allowed assessing presence of target compounds due to carry-over effects. This experiment was conducted in triplicate.

Matrix effects

Evaluation of matrix effects on the ionization response and extraction efficiency was achieved in regards to recommendations published elsewhere (146). A neat solution was fortified at low, medium and high concentration in the initial mobile phase ACN: ammonium formate 10 mM (pH 3.0) buffer (99:1) (a), along with a pool of six urine samples from nicotine-abstinent subjects fortified in triplicate prior to sample treatment (b). By comparing the absolute peak areas of aqueous and urine solutions, matrix effect can be assessed, as reported below (Equation (1)).

$$\% \text{ Matrix effect (ME)} = b/a \qquad (1)$$

Adopting a DS approach, no extraction procedure was required, resulting in interchangeable process efficiency (PE) and matrix effect (ME). Indeed, with this particular sample treatment, ME may only be attributed to ionization of the analytes.

Stability

The effect of storage conditions was evaluated with stability assays designed to mimic the routine analytical throughput of samples. Analyte stability was studied by monitoring the influence of 3 successive freeze and thaw cycles of QC urine samples (n = 3) at low and high concentrations within a week. Since real urine samples were stored at -20 °C in a dark room after collection, QCs were handled likewise and defrosted at ambient temperature 3 consecutive times within a week prior to

LC-MS/MS analysis. The initial integrated peak area was defined as 100%. Similarly, short-term temperature stability was assessed for QCs laying on the bench top at room temperature (21 °C) for 24 h and in the autosampler at 10 ° C for 24h.

C. Results and Discussion

C.1. Method development

UHPLC-MS/MS analyses

Compounds of interest, including nicotine and phase I metabolites along with minor tobacco alkaloids, were selected to highlight recent consumption of tobacco but also to gather comprehensive information on metabolism patterns to help distinguish between smoke and smokeless consumption in a future retrospective study. While phase II glucuronide conjugates of some metabolites may be excreted in abundance in urine, these compounds were not investigated. Indeed, the primary focus was on concentrations of nicotine with potential benefits on sport performance and relevant sensitivity was ensured for all phase I metabolites. Noteworthy, analysis of phase II glucuronide conjugates would require an additional hydrolysis step, which would not have completely allowed a sample treatment as straight-forward as the DS approach. Also, while an abundant literature on LC-MS/MS methods for the quantification of nicotine and selected metabolites in biological fluids has been published, only a handful of publications propose a simple and time-efficient sample treatment followed by fast analysis of a broad range of nicotine metabolites (105, 161, 162).

Indeed, chromatographic and detection conditions were optimized to satisfy identification criteria while allowing a high analytical throughput as favored in dope testing. Accordingly, separation of nicotine, related metabolites and minor alkaloids in urine was found successful with HILIC, using a 50 mm column length and a gradient of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 800 μL/min (Figure 17). Indeed, combining a high flow rate and a short HILIC column with adequate retention properties towards polar molecules and excellent peak shape offered a valuable association of short analysis time while maintaining good resolution.

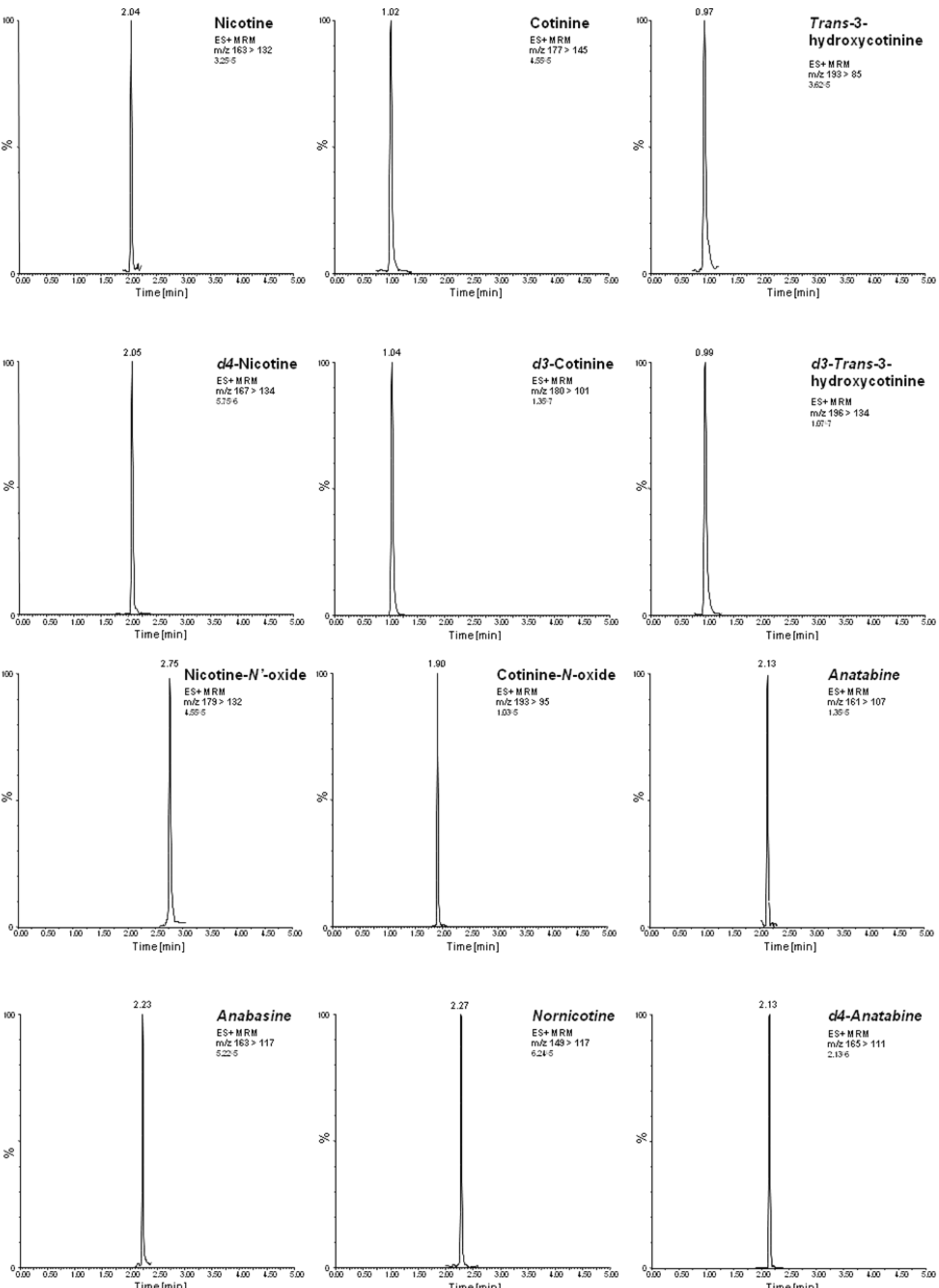


Figure 17. UHPLC-MS/MS chromatogram of a urine specimen containing nicotine, nicotine metabolites and tobacco-related alkaloids at a concentration of 50 ng/mL, with IS spiked at 250 ng/mL for *d4*-nicotine and *d4*-anatabine and 50 ng/mL for *d3*-cotinine and *d3*-*trans*-3-hydroxycotinine.

Interestingly, ionization of target compounds was optimized by use of a mobile phase highly enriched in polar organic solvent, which led to an increase of sensitivity (137). Also, coupling a TQ-MS analyzer to a UHPLC system brought a significant contribution to the enhancement of Signal-to-Noise ratio. Indeed, Multiple Reaction Monitoring (MRM) acquisition mode provided an efficient isolation of characteristic fragment ions for each molecule, resulting in reduced endogenous matrix interferences. Therefore, analytical conditions ensured the suitability of a DS sample treatment for urinary concentrations of nicotine, metabolites and minor alkaloids.

Likewise, triple quadrupole MS/MS parameters were optimized by direct infusion of individual standard solutions. In consequence, compound specific normalized collision energy, cone voltage and dwell time were automatically tuned, producing a high sensitivity fragmentation pattern with a precursor ion response < 10% in abundance (Table 6).

Eventually, repeatability of the retention times (t_R) was assessed by calculating the RSD of each target compound over the set of three validation series, accounting for 21 urine specimens (Table 6). Actually, fluctuations in the chromatographic conditions, including freshly prepared mobile phases, may influence the variability of t_R. Nevertheless, stability of t_R was found satisfactory, as illustrated by a RSD ranging from 0.3 – 1.7% depending on the analyte.

Dilute-and-shoot sample treatment

DS provided an interesting approach to the problem of finding a cost and time efficient sample treatment procedure when dealing with the consistent flow of urine specimens and restricted reporting time particular to dope testing (163-165). Indeed, this simple method involved only limited manipulation of sample, with feasibility for automation which proved efficient for performing large batches of analyses. Coupled with short chromatographic run times allowed by UHPLC, a significant workflow of analysis was achieved. Noteworthy, this method is accepted by WADA, as mentioned in the ISL (6).

Since DS sample treatment is non-selective, as opposed to solid-phase (SPE) or LLE extraction, a variety of matrix compounds may compete with target analytes for ionization and account for substantial ME. Optimization of the dilution factor was carried out to reduce this phenomenon while maintaining sufficient sensitivity. In consequence, a 40-fold dilution appeared to provide an adequate compromise between these two parameters within the dynamic range of the mass analyzer. Also, dilution in ACN allowed improving urine clean-up due to protein precipitation and subsequent centrifugation, proving efficient to diminish potential ME. Therefore, chromatographic column lifetime could be extended and maintenance rate decreased compared to reversed phase chromatography where water is generally used for DS sample treatment.

Eventually, DS sample treatment provided a green alternative to SPE and LLE by requiring a low volume of organic solvent and avoiding addition of chemicals. Regarding the need of a mobile phase highly enriched in ACN for HILIC mode, this parameter was particularly valuable.

C.2. Assay validation

Calibration curves

Considering the pharmacological properties of nicotine and a doping perspective, concentration ranges were determined to comprehend urinary levels relevant for assessing recent consumption (147, 148). Accordingly, great efforts to develop an extensive clean-up procedure of urine samples could be avoided as quantification down to trace levels corresponding to environmental tobacco exposure or end of excretion phase after active consumption was unnecessary. Noteworthy, a fit-for-purpose approach was adopted to determine the dilution factor, which could be lowered to achieve higher sensitivity and address the later problem.

Calibration curves were built with calibration standards at six concentration levels, while evaluating different curves fitting. Referring to the accuracy profiles established over the dosage range, linear least-squares regression with $1/x^2$ weighting was chosen for quantification purpose, with R^2 greater than 0.995 (Figure 18).

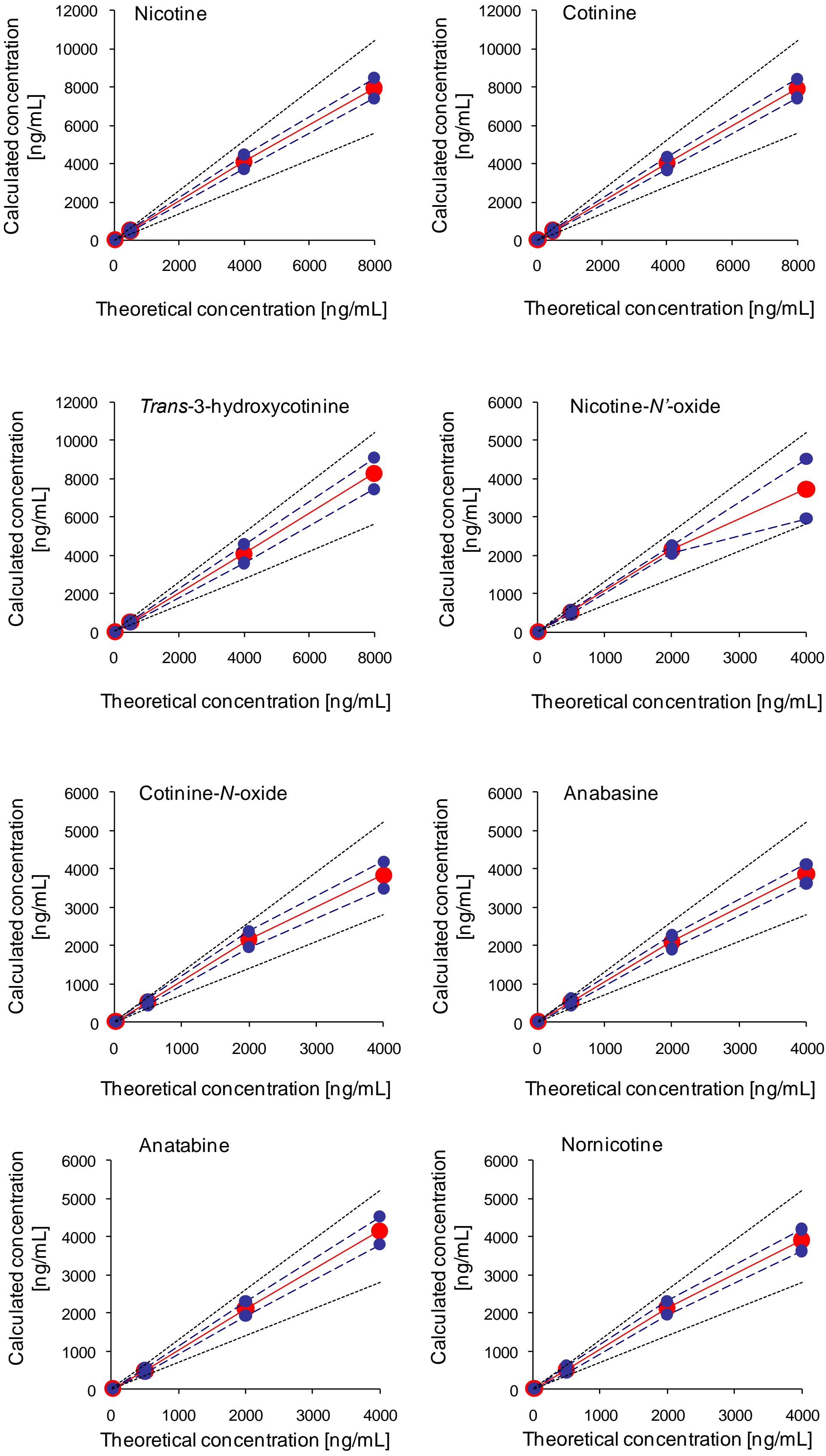


Figure 18. Absolute accuracy profiles for nicotine, nicotine metabolites and tobacco-related alkaloids. The solid line indicates the accuracy and the dashed lines represent the accuracy calculated as confidence interval (145). The dotted lines depict the acceptance limits of ± 30 %.

Repeatability and intermediate precision met the guidelines for bioanalytical method validation over the whole assay range, with RSD values lower than 15% (Table 7). Also, accuracy was found acceptable for all compounds of interest, with measured concentrations within ± 15% of every theoretical concentration. Noteworthy, an 80-fold dilution proved valid for concentrations exceeding the ULOQ.

Molecule	**Concentration (ng/mL)**	**Accuracy (%CV)**	**Precision**	
			Repeatability (%CV)	Intermediate precision (%CV)
nicotine	20	103.7	6.2	6.7
	500	101.1	6.3	8.3
	4’000	102.5	3.8	5.4
	8’000	99.3	3.1	3.8
cotinine	20	101.9	5.5	5.9
	500	101.6	7.5	8.8
	4’000	100.4	2.9	4.7
	8’000	99.1	3.0	3.5
trans-3-hydroxycotinine	20	95.9	5.0	5.0
	500	100.8	7.0	9.3
	4’000	102.1	4.8	7.0
	8’000	103.4	3.9	5.9
nicotine-*N’*-oxide	20	77.7	3.1	4.0
	500	103.6	6.2	6.2
	2’000	107.5	2.4	2.4
	4’000	93.2	3.2	3.2
cotinine-*N*-oxide	20	99.9	6.5	6.8
	500	104.5	6.8	9.1
	2’000	108.1	2.3	6.2
	4’000	95.8	3.9	5.1
anabasine	20	105.5	4.0	4.1
	500	103.8	7.4	9.8
	2’000	104.3	4.8	5.1
	4’000	96.8	2.9	3.6
anatabine	20	104.1	8.1	9.9
	500	97.0	7.5	8.2
	2’000	105.7	4.3	5.5
	4’000	104.0	3.7	5.2
nornicotine	20	97.4	5.2	6.4
	500	104.2	7.6	10.2
	2’000	106.7	4.3	5.2
	4’000	97.8	3.1	4.1

Table 7. Assay validation parameters for nicotine and metabolites (n = 3).

Therefore, direct quantification of target analytes in urine with this UHPLC-MS/MS method was suitable over the assay range.

Eventually, a LOD standing around 1 ng/mL for all compounds could be estimated.

Selectivity

Selectivity evaluation towards interfering endogenous matrix compounds was conducted on 6 different urine specimens from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days. According to triplicate sample treatment followed by UHPLC-MS/MS analysis, no interfering endogenous molecules were highlighted within selected transition windows as ion identification criteria were not met (140). Indeed, retention times, ion transitions and ion ratios parameters observed for matrix components significantly differed from each compound of interest.

Likewise, potential influence of exogenous xenobiotics was assessed relying on routine screening analysis of every urine specimen, searching for over 200 substances appearing on the 2010 Prohibited List and Monitoring Program, followed by sample treatment and UHPLC-MS/MS analysis. Noteworthy, presence of stimulants commonly found in sport and society was investigated, including caffeine and pseudoephedrine. Accordingly, no exogenous xenobiotic highlighted by the screening procedure interfered with target analytes within selected transition windows, referring to the criteria mentioned earlier.

Carry-over

Carry-over was assessed by injecting a blank urine sample following the analysis of the highest calibrator for each compound of interest. This experiment was repeated three successive times. Presence of target analytes was not detected, confirming potential carry-over effects as a negligible phenomenon.

Matrix effect

Comparison of signals detected in urine and in the neat solution highlighted concentration-dependant ion enhancement or suppression effects depending on the compound of interest. Indeed, evaluation of ME indicated ion suppression occurring at low level in the 70 to 98% range due to endogenous matrix compounds competing with target analytes towards ionization. Despite this phenomenon could be expected from a DS approach, the sample treatment method proved fit-for-purpose, as illustrated by the good repeatability of these measurements (RSD < 15%) and the sufficient sensitivity to quantify all substances at the LLOQ. Likewise, all compounds showed substantial ion enhancement at medium and high concentrations, ranging from 104 to 124% and 107

to 125%, respectively, with evidence of good repeatability (RSD < 15%). Noteworthy, ME was efficiently corrected by the deuterated standards at all concentration levels. Therefore, influence of ME on the quantitative analyses was found negligible.

Stability

Analyte stability was evaluated after investigation on the influence of the routine analytical throughput on compounds of interest in QC samples (*n* = 3) at low and high concentrations. Since stability experiments were designed to mimic actual storage and analysis conditions, QCs were preserved at -20 °C in a complete dark environment followed by a series of 3 successive freeze and thaw cycles within a week. Likewise, QC samples were left on the bench top at room temperature (21 °C) for 24 h and in the autosampler at 10 ° C for 24h to assess short-term temperature stability.

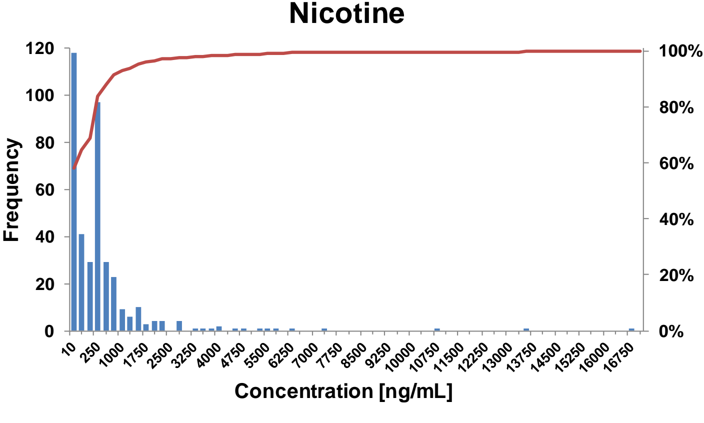
According to minimal variations observed in the peak areas for analyte stability and short-term temperature stability assessments (RSD < 15%), storage conditions adopted during this study were adequate to ensure a high stability of all compounds of interest.

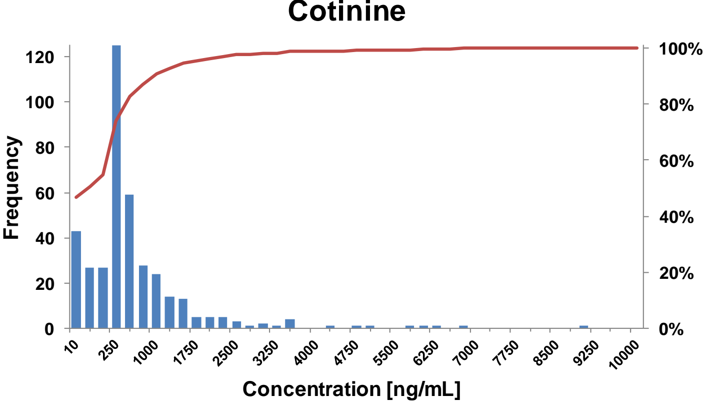
D. Application to doping controls in 2010-2011

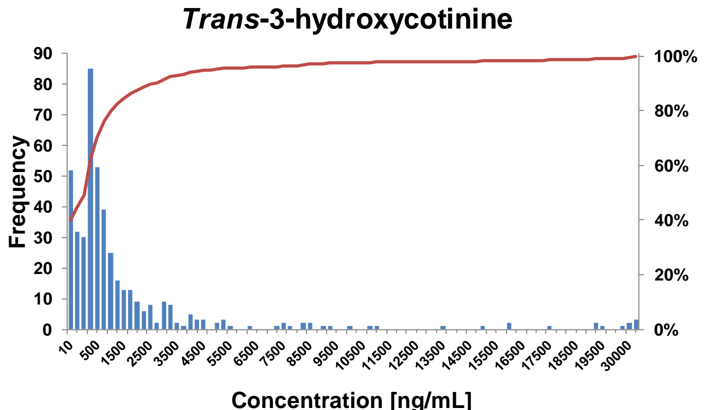
Over a one-year period of time covering 2010 and 2011, every single urine specimen from regular doping controls of 43 different disciplines analyzed by LAD was included in this monitoring (*n* = 2’185). Referring to the 2009 ISL, article 19 of the World Anti-Doping Code and articles 24-27 of the UNESCO Convention against doping in sport, complete removal of identification means and a minimum storage period of three months were ensured prior to initiation of this research (3, 6, 149). Also, approval was received from ADCH to conduct this study.

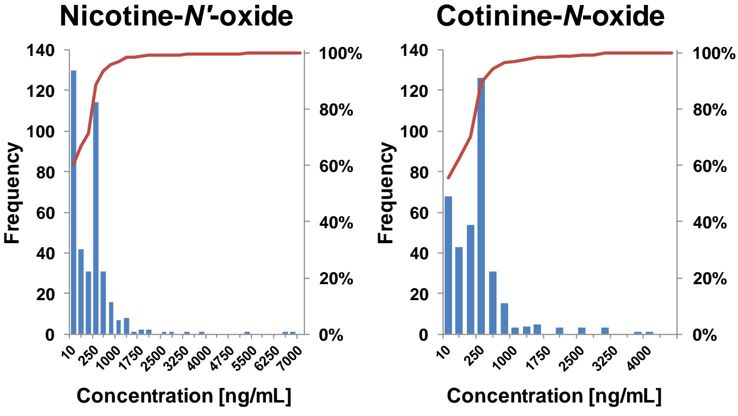
Due to the linear response observed after $1/x^2$ weighting in the assay validation, quantification was performed using calibration standards reduced to LLOQ, medium and ULOQ concentration levels (*k* = 3) and QCs to low and high concentration levels (*k* = 2), prepared each in triplicate. Also, a qualitative designation was assigned to compounds of interest detected in the LOD to LLOQ concentration range, namely traces.

According to the quantitative assays, concentrations distribution of major nicotine metabolites, minor nicotine metabolites and tobacco alkaloids were found to range from LLOQ to 32’223, 6’670 and 538 ng/mL, respectively (Figure 19).


Nicotine
Frequency
Concentration [ng/mL]
0
20
40
60
80
100
120
0%
20%
40%
60%
80%
100%
10
250
1000
1750
2500
3250
4000
4750
5500
6250
7000
7750
8500
9250
10000
10750
11500
12250
13000
13750
14500
15250
16000
16750


Cotinine
Frequency
Concentration [ng/mL]
0
20
40
60
80
100
120
0%
20%
40%
60%
80%
100%
10
250
1000
1750
2500
3250
4000
4750
5500
6250
7000
7750
8500
9250
10000


Trans-3-hydroxycotinine
Frequency
Concentration [ng/mL]
0
10
20
30
40
50
60
70
80
90
0%
20%
40%
60%
80%
100%
10
500
1500
2500
3500
4500
5500
6500
7500
8500
9500
10500
11500
12500
13500
14500
15500
16500
17500
18500
19500
30000


Nicotine-N'-oxide
Frequency
Concentration [ng/mL]
0
20
40
60
80
100
120
140
0%
20%
40%
60%
80%
100%
10
250
1000
1750
2500
3250
4000
4750
5500
6250
7000
Cotinine-N-oxide
Frequency
Concentration [ng/mL]
0
20
40
60
80
100
120
140
0%
20%
40%
60%
80%
100%
10
250
1000
1750
2500
3250
4000

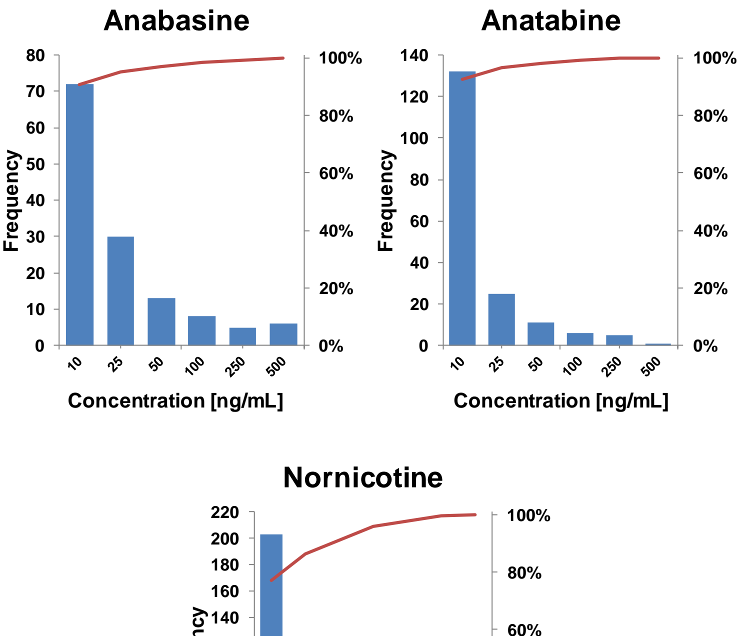


Figure 19. Concentrations distribution for nicotine, its metabolites, minor tobacco alkaloids and tobacco-related alkaloids. The solid red line indicates the cumulative percentage.

Traces of major nicotine metabolites, minor nicotine metabolites and minor tobacco-related alkaloids accounted for 17.9 to 20.5%, 16.5 to 17.9% and 6.1 to 16.2% of urine specimens, respectively (Table 8).

Analyte	Concentration range		
	$x \geq$ LOD	$x \geq$ LLOQ	**Active exposure**
nicotine	17.9%	12.5%	9.3%
cotinine	18.0%	16.1%	13.6%
trans-3-hydroxycotinine	20.5%	18.1%	15.3%
Cumulative exposure	**23.0%**	**18.3%**	**15.3%**
nicotine-*N*′-oxide	17.9%	11.9%	10.0%
cotinine-*N*-oxide	16.5%	13.4%	11.4%
anabasine	6.1%	2.8%	1.5%
anatabine	8.2%	2.2%	1.1%
nornicotine	16.2%	6.9%	4.2%

Table 8. Prevalence of urine samples exposed to nicotine, major nicotine metabolites or minor tobacco alkaloids depending on the concentration range (*n* = 2’185).

Actually, at least one of the five different metabolites and three related tobacco alkaloids was detected in 23% of samples. Thus, exposure to nicotine, either due to active consumption or environmental tobacco smoke, concerned approximately twenty-three athletes out of a hundred. Considering a worldwide smoking prevalence of approximately 25%, as reported by the WHO, and huge progress towards implementation of a smoke-free environment, such findings support the potential use of nicotine in sport with a specific purpose (68, 166). When focusing on sports with a significant number of samples submitted to dope testing, cumulative exposure to nicotine metabolites and tobacco alkaloids was found to range between 26 and 56% of urine specimens for ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics (Table 9).

	Discipline	*n*	Active exposure
Winter sports	snowboarding	16	43.8%
	ski jumping	10	40.0%
	alpine skiing	38	36.8%
	ice hockey	96	36.5%
	bobsleigh	78	30.8%
	cross country skiing	34	23.5%
	biathlon	38	18.4%
	figure skating	41	17.1%
Ball games	beach volley	7	57.1%
	American football	19	55.6%
	floorball	54	38.9%
	rugby	25	28.0%
	basketball	24	20.8%
	football	201	19.4%
	handball	44	15.9%
	volleyball	35	14.3%
	tennis	31	12.9%
Individual sports	gymnastics	16	37.5%
	wrestling	31	32.3%
	artistic gymnastics	27	25.9%
	athletics	461	8.7%
	boxing	12	8.3%
Endurance sports	track cycling	15	20.0%
	road cycling	131	12.2%
	mountain bike cycling	18	5.6%
	rowing	21	4.8%
	triathlon	35	2.9%
	swimming	53	1.9%

Table 9. Prevalence of active nicotine exposure in selected sports depending on the type of discipline.

Likewise, above-LLOQ levels of major nicotine metabolites, minor nicotine metabolites and minor tobacco-related alkaloids were quantified in 12 to 18%, 12 to 13% and 2 to 7% of urine specimens, respectively. At least one of the five different metabolites and three related tobacco alkaloids was measured in this concentration range in 18% of samples. As such, approximately eighteen athletes out of a hundred were exposed to smoke or ST or ETS within the last 3 days before doping control (135). Regarding the specific disciplines mentioned previously, recent cumulative exposure concerned 20 to 56% of urine specimens.

Eventually, measuring prevalence of nicotine consumption prior to and/or during sport practice was carried out by hypothesizing conservative concentration limits for active exposure (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-*N'*-oxide and cotinine-*N*-oxide), as previously depicted (135, 151). Likewise, since extensive exposure to second-hand smoke shortly before sport practice was extremely unlikely, contribution of ETS to such levels of concentration could be excluded. Thus, referring to those concentration ranges and considering major nicotine metabolites, minor nicotine metabolites and minor tobacco-related alkaloids, consumption of nicotine associated to sport training or competition was highlighted in 9 to 15% and 10 to 11% of urine specimens, respectively. Noteworthy, at least one of these compounds of interest was present in such concentrations in 15% of samples. Accordingly, about fifteen athletes out of a hundred were considered as active nicotine consumers in competition, regardless of the sport discipline. These figures should be carefully interpreted as the statistical significance is not ensured for sports disciplines with low numbers of samples and as they correspond to the geographical area of Switzerland only. As a consequence, the true prevalence may be either underestimated or overestimated. However, while such statistics may appear lower than smoking prevalence in society, putting emphasis on winter sports, as well as a couple ball sports and individual sports, indicated a consumption prevalence comparable, if not far superior, to regular recreational use. Indeed, active exposure close to or during games and races has been highlighted in up to 57% of urine specimens, depending on the sport.

Whether this significant prevalence is the consequence of a social trend within particular communities of athletes and/or an attempt to increase performances in specific disciplines remains unknown. Nevertheless, with respect to the detrimental effects of smoking on the respiratory system, these figures bring a significant support to the hypothesis of ST use, whether the primary purpose is performance enhancement or not (154-157). At present, a more definite number for prevalence of ST use could not be determined as there is no reported clinical study proposing a metabolic-based approach to distinguish between different forms of nicotine consumption. Nevertheless, a retrospective study after future development of biostatistical tools appears feasible

as a large number of compounds related to the metabolism of nicotine or tobacco alkaloids have been quantified.

E. Conclusion

This one-year monitoring study confirmed the alarming IC exposure levels previously described for ice hockey and highlighted a similar situation in several other sport disciplines. Due to the significance of the phenomenon, including nicotine to the Monitoring Program would allow determining geographical variations and assessing whether nicotine consumption is growing and durable or, on the contrary, stagnant, if not decreasing, and ephemeral.

Considering a public health perspective, a preventive rather than repressive attitude in anti-doping may be an interesting and innovative approach to tackle this alarming phenomenon. Indeed, while the inclusion of nicotine to the Prohibited List may reduce consumption of this molecule as a performance enhancer, actions and policies should primarily be elaborated in order to prevent the initiation and development of drug addiction during sport practice and its pursuit after a professional career. This holds particularly true as young generations of athletes tend to follow the steps of their older peers. While the frontier between recreational consumption and use for doping purpose is difficult to ascertain with social drugs, including caffeine or tetrahydrocannabinol (THC), toxicity of tobacco products is responsible for disastrous health effects greatly amplified by persistent addiction issues.

Therefore, WADA and sport federations should evaluate the inclusion of nicotine to the Prohibited List and/or Monitoring Program in order to bring not only control on a potential doping agent, but also an innovative and key element to developing a more preventive approach of fight against doping. Indeed, an interesting step towards limitation and education on a global public health threat responsible for an extremely harmful burden of disease could be initiated within the sports community.

This study was one of the key arguments to include nicotine to the Monitoring Program in 2012.

III.3.3. Distinction between smoke and smokeless tobacco consumption

A. Introduction

According to the previous researches, there was a need for developing bioanalytical, statistical and probabilistic tools to distinguish between smoke and ST consumption, whether for identification of users or for specific prevalence measurements. Therefore, a clinical study on the metabolism of nicotine, tobacco-specific nitrosamines and tobacco combustion products was designed to answer

these questions (protocol in Appendix V.3). Approval was received by the Human Research Ethics Committee of Vaud to conduct this study.

The project was conducted in collaboration with the Polyclinique Médicale Universitaire (PMU) and the Centre de Recherche Clinique (CRC) for medical supervision and assistance in the collection of biological samples, as well as the Institut de Santé au Travail (IST) for smoking in a controlled air-flow exposure room.

B. General plan

In a first step, healthy male volunteers (n = 21) aged 20 to 30 years old who are occasional smokers were recruited by advertisement made at CHUV, the University of Lausanne (UNIL) and the Ecole Polytechnique Fédérale de Lausanne (EPFL).

A preliminary briefing given by the investigator (François Marclay) covering the different aspects of the study was held for the volunteers who indicated their willingness to participate in this clinical study. An information sheet was given to the subjects as well as a consent form. In order to allow a sufficient reflection time, volunteers were asked to return this document within 3 days following the briefing. As soon as the consent was returned duly signed, volunteers were invited to the inclusion visit and underwent a medical examination conducted by a doctor of the PMU. Inclusion and exclusion criteria were checked using a questionnaire and a subject number was randomly assigned to each participant (details in Appendix V.3). This number was reported in the case observation form and only the investigator and his staff, all subject to professional secrecy, had access to the personal data of the volunteers.

The protocol to monitor the metabolism of nicotine, tobacco-specific nitrosamines and tobacco combustion products is summarized in Table 10.

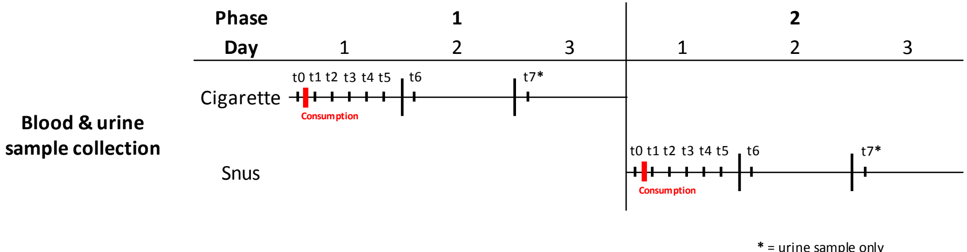


Table 10. Exposure to nicotine and sample collection protocol for the clinical study.

In order to guarantee the absence of tobacco-related compounds in the body, the participants were asked not to consume nicotine during the 3 days prior to and after the start of each phase of the study.

On day 1 of the first phase, a standard cigarette (Parisienne Jaune) of 0.8 g tobacco containing 0.7 mg of nicotine was smoked by the volunteers in a controlled environment at the IST. A MoNIC

(Moniteur de NICotine) badge had also been pinned to each participant's garment in order to measure the extent of self-induced second-hand exposure during this phase (167). Blood samples were collected at t_0 = 0h (before exposure), t_1 = 2h, t_2 = 4h, t_3 = 6h, t_4 = 8h, t_5 = 10h and t_6 = 24h after intake. Similarly, urine samples were collected at t_0 to t_6 and t_7 = 48h after consumption. Then, a week break was observed to ensure complete removal of substances of interest from the body.

On day 1 of the second phase, a 24 g bag of snus tobacco containing 8mg of nicotine was used by the volunteers for a period of 30min at the CRC. The blood and urine samples were collected similarly to the first phase.

In practice, blood samples (minimum 5mL of serum) were collected in duplicates directly by the CRC medical staff from t_0 to t_6. One of each duo of samples was successively centrifuged, aliquoted and frozen at -20 °C until being analyzed while blood parameters of the other one were determined using a Sysmex blood analyzer before centrifugation and freezing. Urine samples (minimum 50mL) were collected by the volunteers in containers provided for this purpose from t_0 to t_7. In order to normalize the concentrations of the compounds of interest, creatinine was measured using a clinical chemistry rapid assay and urine specific gravity using a refractometer. The pH was also calculated alongside. Afterwards, the urine samples were aliquoted and frozen at -20 °C until analysis.

This project having not been completed yet, the key linking the random subject number to the participant will be destroyed at the end of the study to ensure complete and definitive anonymisation of the samples.

C. Bioanalytical strategy

This project was subdivided into several sub-sections to prioritize the parts offering a good balance between promising value for discrimination and compatibility with routine doping analyses (Table 11).

Analyte	Matrix	Discrimination potential	Doping analyses compatibility	Priority
nicotine and metabolites	urine	medium	high	**1**
	blood	medium	high	**2**
tobacco combustion products	urine	high	low	**3**
	blood	high	low	**4**
tobacco-specific nitrosamines	urine	low	medium	**5**
	blood	low	medium	**6**

Table 11. Summary of the different analytical parts of the clinical study on smoke and smokeless consumption. An order of priority has been established for each part depending on the compatibility with routine analytical doping tests and the discrimination potential between each population of user.

As an end goal to this clinical study, the discrimination potential through the identification of biomarkers for distinguishing between smoke and ST consumption was naturally of outmost importance. Since one form implies tobacco pyrolysis, as opposed to the other one, combustion products (hydroxynapthol, hydroxypyrene and hydroxyfluorene and related glucuronides) were very likely to be found in significantly larger quantities. Therefore, the hypothesis upon which these molecules offered the highest potential was relatively self-explanatory and related to the very distinct physical natures of each form of use. As absorption routes differ for both forms of consumption, differences in the pharmacokinetic of nicotine metabolism (nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide, cotinine-*N*-oxide and related glucuronides) and minor tobacco alkaloids (anabasine, anatabine and nornicotine) could be reasonably hypothesized. Eventually, the curing process of smoke and ST being different, variations in the quantity of tobacco-specific nitrosamines (in particular 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and glucuronide) absorbed could be expected. Nevertheless, due to the large intra-variability within batches of production and inter-variability between brands reported in the literature, these molecules were attributed the lowest potential for discrimination.

A higher priority was given to analytes which would require a sample preparation and instrumentation fitting with routine doping analyses. The possibility to include biomarkers for the distinction between smoke and ST consumption to existing urine or blood analyses run on a weekly basis was considered a huge asset. Since anti-doping requires cost- and time-efficient procedures, there is a constant push towards the synergy of different methods for the rapid analysis of large numbers of molecules. According to the literature, the most recent approaches for nicotine and metabolites analysis employ DS or mixed-mode cation exchange SPE (MCX) sample preparations, whether with or without an hydrolysis step for the deconjugation of glucuronated metabolites, before injection on a UHPLC-MS/MS system. These techniques being widely used in routine anti-doping analyses, nicotine and metabolites were considered most compatible compared to the other molecules of interest. The analysis of tobacco-specific nitrosamines implies similar methods but the demand for a higher sensitivity due to lower concentrations in blood and urine and the need for a hydrolysis step during sample preparation made these compounds slightly less compatible with routine doping analyses. Eventually, tobacco combustion products require a more challenging and extensive sample preparation due to their extremely low abundance in biological samples. Accordingly, a dedicated analytical procedure is needed. Also, as combustion products are not tobacco-specific, the analysis of nicotine and metabolites is a prerequisite to screen for tobacco users. Therefore, these molecules were considered as the least compatible with the routine laboratory workflow.

WADA pressed the argument that the issue of distinguishing between different origins of a substance with a prohibited and a non-prohibited status is a key objective. In accordance with the Code, priority should be given as much as possible to finding specific markers of the prohibited route of administration to unambiguously demonstrate the origin of the product. In these views, WADA stated that even if the objectives of this study were well identified, the order of priority did not appear in line with an optimized and practical outcome in the anti-doping field. From a fundamental science perspective, the author agrees with the philosophy described by WADA. Nevertheless, since anti-doping is practical science by nature, method developments should be compatible with routine analysis requirements or, at least, offer a reasonable two-steps solution comprising routine and confirmation analyses. Considering the bioanalytical challenges discussed in this section, routinely screening for combustion products is unimaginable, thus making it a confirmatory analysis. As a matter of balance, and to use the words of WADA, giving priority to nicotine and metabolites was found to be more in line with an optimized and practical outcome in the anti-doping field due to the theoretical possibility of integration to routine protocols and the potential discriminating power.

In summary, priority was given to the analysis of nicotine and metabolites, followed by combustion products and tobacco-specific nitrosamines.

Eventually, urine is still the primary matrix used in dope testing and came first in order of priority compared to blood even though the latter is more informative in terms of pharmacokinetics.

D. Part I: Nicotine and metabolites in urine

D.1. Experimental

The previous analytical method for the simultaneous determination and quantification of nicotine, its four main unconjugated metabolites and minor tobacco alkaloids in urine involved DS sample treatment followed by UHPLC-TQ-MS/MS analysis in HILIC mode.

For the present study, the 10 ng/mL LLOQ for the compounds of interest offered by this approach was found to be a limitation as concentration levels reached after the consumption of a single cigarette or snus pouch were expected to be in the low range. While this analytical method would be satisfactory for measuring peak concentrations, samples shortly after nicotine consumption or towards excretion completion might have been problematic. Therefore, as superior sensitivity and selectivity were required, a MCX sample preparation was developed to replace the DS procedure. Nevertheless, the LLOQ reported in the literature for the glucuronated metabolites of nicotine, cotinine and *trans*-3-hydroxicotinine would not have been fit-for-purpose (162, 168). In particular, the measurement of cotinine-glucuronide may have been an issue. As a consequence, a hydrolysis step was introduced prior to MCX extraction to recover both free and conjugated metabolites (total fraction) under their free form, while a second aliquot of the same sample was directed extracted by

MCX to obtain the free metabolites alone (free fraction). A simple subtraction between the total and the free fractions allowed obtaining the concentrations of the glucuronated metabolites. A pentafluorophenylpropyl (F5) stationary phase was also chosen in order to increase the selectivity between nicotine and anabasine as these molecules have the same molecular weight and very close chromatographic behaviors in HILIC mode.

Sample preparation

An aliquot of urine (2 mL) was spiked with 40 μL of deuterated internal standard (IS) solution (*d4*-nicotine, *d3*-cotinine, *d3*-*trans*-3-hydroxycotinine and *d4*-anatabine at 2.5 μg/mL in MeOH) and split in two fractions of equal volume. 1mL of 0.2M phosphate buffer (pH 6.7) and 50 μl of β-glucuronidase were added to the first aliquot prior to incubation at 50 °C overnight in a thermostated water bath. Afterwards, both fractions were acidified with 1 mL of 2% aqueous formic acid to adjust the pH to ~2.5. After vortex mixing, the aliquots were centrifuged for 5 min at 2500 rpm and subjected to SPE. Briefly, Oasis® MCX cartridges ($3cm^3$, 60mg, Waters, Milford, USA) were conditioned with 1 mL of MeOH followed by 1 mL of 2% aqueous formic acid. The samples were loaded onto the cartridges prior to a washing step with 1 mL of 2% aqueous formic acid and 250 μL of MeOH. Analytes were eluted with 1 mL of 5% (v/v) ammoniated MeOH. 100 μL of 1% HCl in MeOH were added in order to improve nicotine recovery. After vortex mixing, the samples were evaporated to dryness under a gentle air stream at 40 °C and reconstituted in 150 μL of 10 mM ammonium acetate (pH 5.0): MeOH (70:30, v/v) prior to UHPLC-TQ-MS/MS injection.

Efficiency of β-glucuronidase hydrolysis

Aliquots of urine (1 mL) were spiked with 20 μL of deuterated internal standard IS solution and 20 μL of a standard solution containing glucuroconjugated compounds (nicotine-N-β-glucuronide, cotinine-N-β-D-glucuronide and *trans*-3-hydroxycotinine-O-β-D-glucuronide at 10 μg/mL in MeOH). 1mL of 0.2M phosphate buffer (pH 6.7) and 50 μl of β-glucuronidase were added prior to incubation at 37 °C for different times (2, 4, 6, 8, 10 and overnight) in a thermostated water bath. Afterwards, the samples were acidified with 1 mL of 2% aqueous formic acid to stop the hydrolysis and adjust the pH to ~2.5 prior to SPE extraction and UHPLC-TQ-MS/MS analysis. Each sample was prepared in triplicate.

UHPLC conditions

Separation was carried out on an Acquity UPLC System (Waters, Milford, USA) with a Supelco Ascentis Express F5 column (2.1 x 100 mm, 2.7 μm, Sigma-Aldrich, Buchs, Switzerland). Column and

autosampler tray temperatures were set at 30 °C and 10 °C, respectively. Mobile phase consisted of 10 mM ammonium acetate (pH 5.0) buffer (A) and MeOH with 0.001% formic acid (B) with a flow rate set at 250 μL/min. Initial mobile phase was 65% A decreased linearly to 50% over 4.5 min and then to 30% at 4.51 min. The column was washed during 0.3 min and mobile phase increased back to 65% to re-equilibrate the system for 0.7 min. The total run time was 5.5 min. Injection volume was fixed at 10 μL in full loop mode.

Triple Quadrupole-MS parameters

Analyses were performed using a Waters Xevo™ TQ-S triple quadrupole mass spectrometer equipped with an ESI source operating in positive mode. MS operating conditions were set as follows: desolvation gas flow set at 600 L/h at a temperature of 550 °C, capillary voltage at 3.0 kV in positive mode, cone voltage and collision energies optimized for each compound (Table 12). The source temperature was 150 °C, the cone gas flow was set to 150 L/h and the collision gas flow was 0.15 mL/min.

Molecule	MRM transition (m/z)*	Collision energy (eV)	Cone voltage (V)	t_R (min)
nicotine	163 → **132**, 117, 84	15	18	2.27
d4-nicotine	167 → 136, **134**, 121	20	20	2.28
cotinine	177 → **146**, 98, 80	16	20	2.04
d3-cotinine	180 → 146, **101**, 81	20	20	2.04
trans-3-hydroxycotinine	193 → **134**, 86, 80	20	16	1.36
d3-*trans*-3-hydroxycotinine	196 → **134**, 89, 80	20	20	1.36
nicotine-*N′*-oxide	179 → **132**, 130, 117	18	20	2.29
cotinine-*N*-oxide	193 → 134, **96**, 79	18	14	1.16
anabasine	163 → 146, 130, **117**	20	40	2.76
anatabine	161 → 144, 117, **107**	18	26	2.36
d4-anatabine	167 → 148, 132, **111**	20	20	2.35
nornicotine	149 → 132, **117**, 106	20	34	1.95

*Quantification ion transitions are in bold.

Table 12. MRM parameters and retention times of the analytes.

Identification criteria

Identification criteria were defined according to the WADA Technical Document addressing qualitative assays, as described in the one-year monitoring study (140).

Method validation

Calibration curves

The method validation approach was a replica of the one-year monitoring study, let aside the calibration which was established over the 1-500 ng/mL concentration range, or on a more limited range depending on the analytes. Noteworthy, as the hydrolysis step was avoided for the calibrator standards, the volumes were divided by two, corresponding to 1 mL of urine and 20 μL of IS. Alternatively, the validator standards underwent the complete MCX sample preparation described previously to assess the influence of this hydrolysis process.

Selectivity

Selectivity assessment of interfering endogenous matrix compounds within selected tolerance windows was carried out as a replica of the approach adopted in the one-year monitoring study.

Carry-over

Likewise, carry-over experiments were conducted as in the one-year monitoring study.

Matrix effects

Evaluation of matrix effects on the ionization response and extraction efficiency was achieved similarly to the study on nicotine consumption during the 2009 IIHF World Championships.

Stability

Again, the effect of storage conditions was evaluated as in the one-year monitoring study.

D.2. Results and Discussion

Method development

UHPLC-MS/MS analyses

Target compounds, including nicotine, metabolites and related glucuronides along with minor tobacco alkaloids, were selected to gather a wide spectrum of information on metabolism patterns after smoke and ST consumption. With pharmacokinetics as a primary focus, phase II glucuronide conjugates were also investigated as some metabolites constitute a significant part of the total urinary excretion. In order to ensure fit-for-purpose sensitivity in the measurement of the glucuronated compounds, each sample was split in two fractions to be analyzed after MCX sample treatment or hydrolysis followed by MCX sample treatment. While not as straight-forward and cheap as DS, this approach offers efficient sample clean-up, which is particularly important for quantification purposes.

As described in the previous studies, chromatographic and detection conditions were optimized to satisfy identification criteria while allowing a high analytical throughput as favored in dope testing. Separation of nicotine, related phase I metabolites and minor alkaloids in urine was found successful with the F5 stationary phase, using a 100 mm column length and a gradient of 10 mM ammonium acetate (pH 5.0) buffer (A) and MeOH with 0.001% formic acid (B) with a flow rate set at 250 µL/min (Figure 20). Relying on a F5 column offered interesting retention properties towards polar molecules, allowing increasing the selectivity between nicotine and anabasine as compared to HILIC, while maintaining very good peak shape. The recent release of the Express line of columns dedicated to UHPLC, as opposed to the regular line of LC columns compatible with UHPLC systems, allowed a very significant reduction of the total analysis time compared to the literature (101, 162, 168). On a further note, the lower flow rate and avoiding the use of ACN as the primary solvent constituting the mobile phase was also a greener alternative to HILIC.

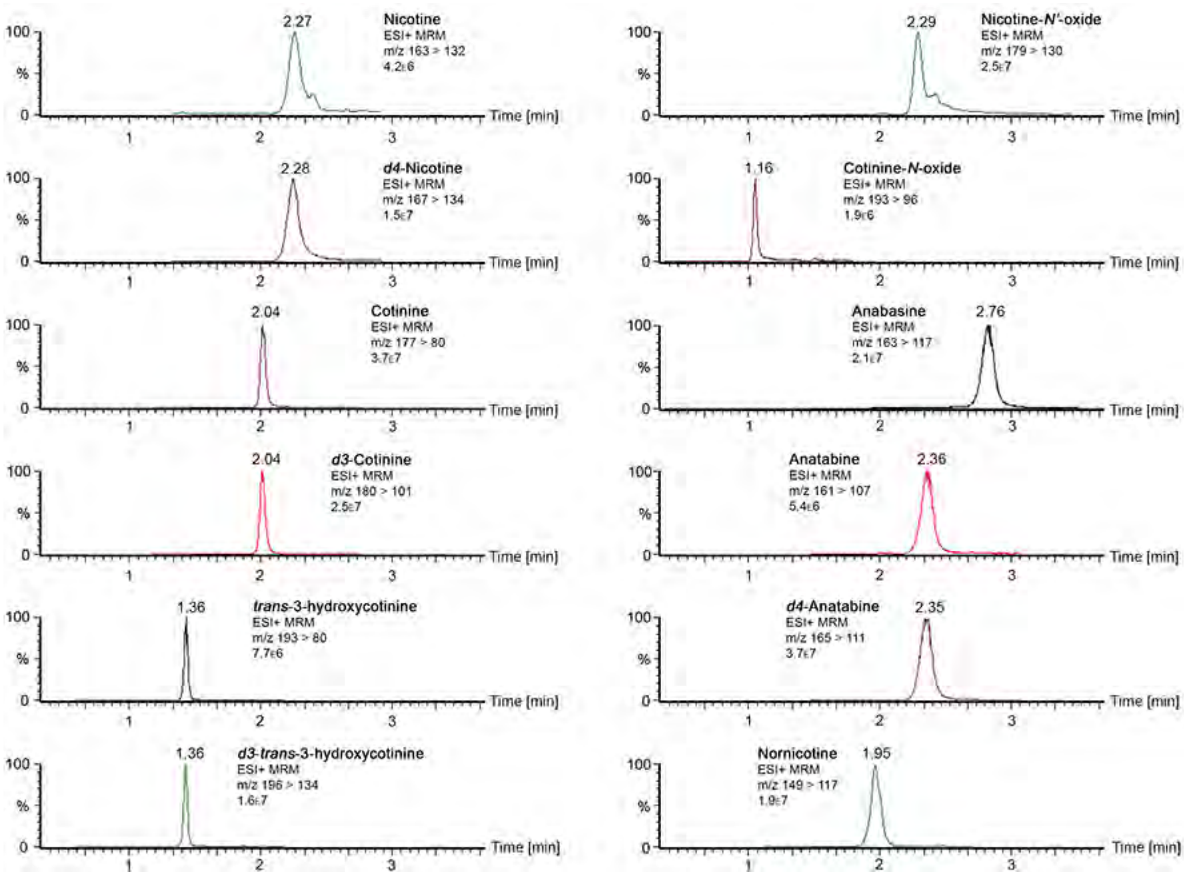


Figure 20. UHPLC-MS/MS chromatogram of a urine specimen containing nicotine, nicotine metabolites and tobacco-related alkaloids at a concentration of 25 ng/mL, with IS spiked at 50 ng/mL for *d4*-nicotine, *d3*-cotinine, *d3*-*trans*-3-hydroxycotinine and *d4*-anatabine.

Triple quadrupole MS/MS parameters were optimized in the previous study to obtain compound specific normalized collision energy, cone voltage and dwell time and produce a high

sensitivity fragmentation pattern. Since the same instrument was used for this research, the MS/MS parameters were retrieved from the previous study (Table 12).

Eventually, repeatability of the retention times (t_R) was assessed by calculating the RSD of each target compound over the set of three validation and calibration series, accounting for 27 urine specimens (Table 12). Even though fluctuations in the chromatographic conditions, including freshly prepared mobile phases, may influence the variability of t_R, the stability was found satisfactory with a RSD ranging from 0.2 – 1.3% depending on the analyte.

MCX sample treatment

MCX sample treatment was a very significant improvement in terms of sensitivity and selectivity compared to both DS and LLE. While not as cost and time efficient as DS sample treatment, the preparation of a set of 48 samples could be achieved within 3h, allowing dealing with the large volume of samples collected during the clinical study in a short period of time.

Since the dilute-and-shoot sample treatment previously used was non-selective, SPE on MCX cartridges allowed filtering out most matrix compounds that may cause selectivity issues and compete with target analytes for ionization and/or result in substantial ME. Extracts were found to be very clean and the MCX procedure allowed a ~6.5-fold concentration of compounds of interest, which was an interesting gain in sensitivity compared to dilute-and-shoot. Also, chromatographic column lifetime was found to be notably extended and maintenance rate decreased with the improved urine clean-up, even without the use of a pre-column.

RE of free metabolites ranged from 87.6 to 101.1% depending on the analyte, with evidence of good repeatability (RSD < 10%), and showed only slight dependency on the concentration level (Table 13).

Molecule	**Concentration (ng/mL)**			**Recovery (%)**			**RSD (%)**		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
nicotine	1	250	500	97.8	96.4	97.2	5	2	4
cotinine	1	250	500	99.1	98.7	101.1	4	3	3
trans-3-hydroxycotinine	1	250	500	94.2	95.5	97.3	6	4	7
nicotine-*N'*-oxide	2.5	50	100	90.6	91.1	92.9	4	7	2
cotinine-*N*-oxide	2.5	50	100	87.6	89.5	90.2	8	4	3
anabasine	1	100	250	99.2	100.8	101.1	3	4	7
anatabine	1	250	500	96.7	95.3	97.4	6	5	2
nornicotine	1	250	500	98.5	98.6	95.3	7	2	8

Table 13. Recovery and related RSD of nicotine, related metabolites and minor tobacco alkaloids at low, medium and high concentrations (n = 3).

Also, as MCX is gaining popularity in dope testing, extraction of nicotine and metabolites could potentially be combined with other prohibited substances (169). According to the previous study and since the routine procedure at LAD relies on DS sample preparation, nicotine, major phase I metabolites and minor tobacco alkaloids can be included to the screening method. In a second step, these compounds and major phase II metabolites could be integrated to the MCX sample clean-up used at LAD for confirmatory analysis of basic and neutral compounds (169). Therefore, MCX should be regarded as a confirmatory method in the perspective of distinguishing forms of tobacco consumption.

Efficiency of β-glucuronidase hydrolysis

As previously reported in the literature, the hydrolysis of the three glucuronides was >90% within the first 6h and complete overnight at 37°C as confirmed by UHPLC-TQ-MS/MS analysis (data not shown) (101).

Assay validation

Calibration curves

Considering the pharmacological properties of nicotine, concentration ranges were determined as a compromise between the urinary levels expected after consumption of a single dose of smoke or ST and the analytical capabilities.

Calibration curves were built with calibration standards at five concentration levels, while evaluating different curves fitting. Referring to the accuracy profiles established over the dosage range, linear least-squares regression with $1/x^2$ weighting was chosen for quantification purpose, with coefficients of determination (R^2) greater than 0.995 (Figure 21).

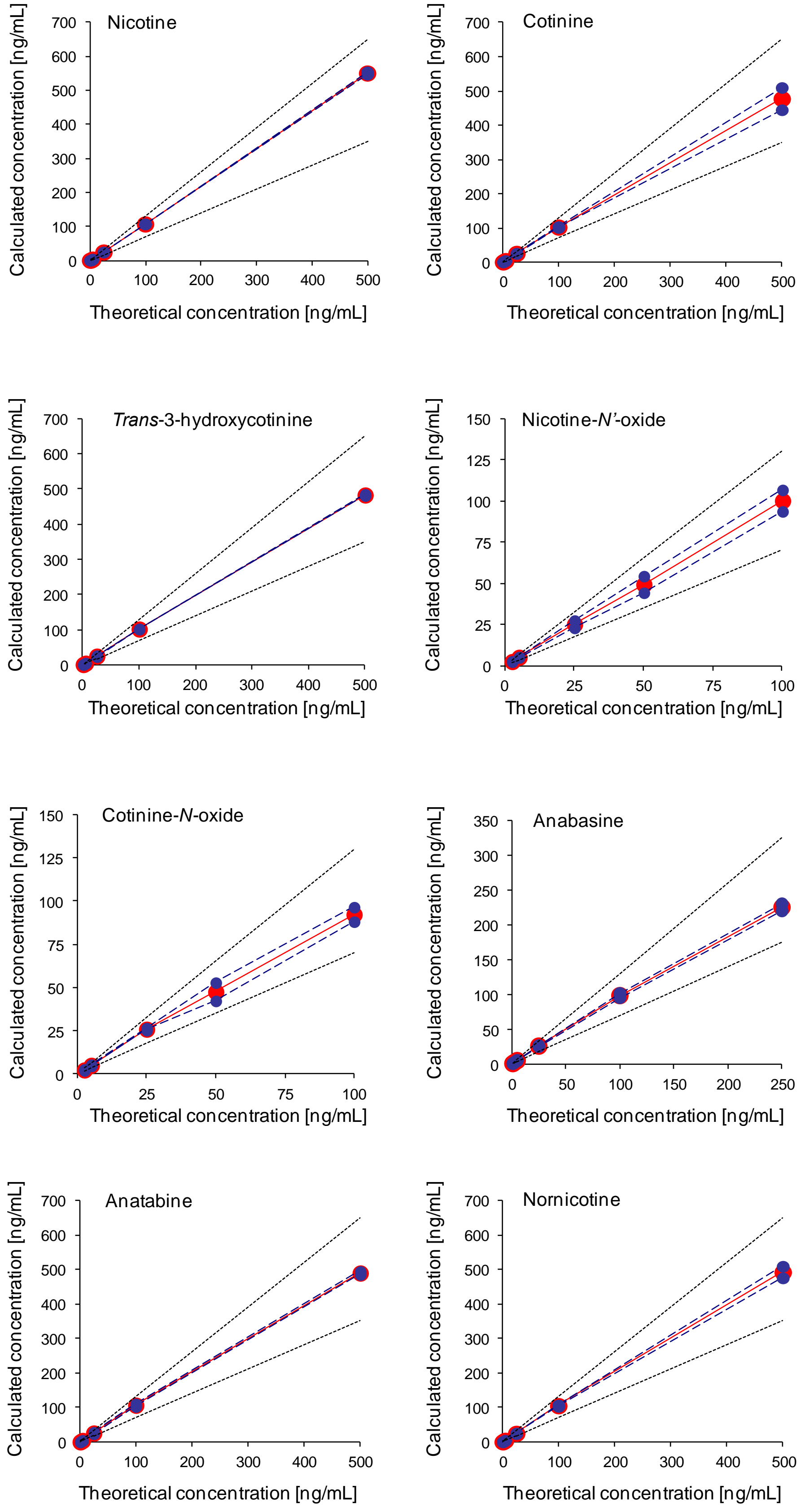


Figure 21. Absolute accuracy profiles for nicotine, nicotine metabolites and tobacco-related alkaloids. The solid line indicates the accuracy and the dashed lines represent the accuracy calculated as confidence interval (145). The dotted lines depict the acceptance limits of ± 30 %.

Repeatability and intermediate precision met the guidelines for bioanalytical method validation over the whole assay range, with RSD values lower than 10% (Table 14). Also, accuracy was found acceptable for all compounds of interest, with measured concentrations within ± 10% of every theoretical concentration. The criteria defined by the guidelines were also met for validator standards which underwent hydrolysis with RSD values lower than 15% for repeatability and intermediate precision, and with measured concentrations within ± 15% of every theoretical concentration (data not shown). Therefore, the influence of hydrolysis was found negligible.

Molecule	Concentration (ng/mL)	Accuracy (%CV)	Precision	
			Repeatability (%CV)	Intermediate precision (%CV)
nicotine	1	100.0%	1.9%	2.1%
	5	96.2%	1.0%	1.2%
	25	96.2%	0.3%	0.3%
	100	100.3%	0.1%	0.1%
	500	101.9%	0.4%	0.4%
cotinine	1	100.4%	6.6%	6.7%
	5	99.1%	1.8%	2.0%
	25	104.1%	1.0%	1.0%
	100	102.7%	0.7%	0.7%
	500	95.5%	3.6%	3.7%
trans-3-hydroxycotinine	1	101.1%	7.1%	7.1%
	5	96.8%	1.3%	1.3%
	25	99.5%	3.2%	3.4%
	100	101.8%	0.2%	0.2%
	500	96.5%	0.1%	0.2%
nicotine-*N'*-oxide	2.5	100.7%	2.6%	2.6%
	5	102.7%	1.1%	1.6%
	25	101.0%	5.3%	5.3%
	50	99.0%	5.2%	5.7%
	100	100.6%	3.7%	3.9%
cotinine-*N*-oxide	2.5	98.1%	7.6%	7.2%
	5	102.8%	3.6%	4.0%
	25	104.1%	1.0%	1.4%
	50	95.3%	6.3%	6.1%
	100	92.4%	2.5%	2.4%
anabasine	1	97.7%	3.6%	4.8%
	5	105.6%	2.7%	2.7%
	25	104.1%	1.1%	1.4%
	50	98.5%	1.3%	1.7%
	250	90.0%	1.1%	1.2%
anatabine	1	94.2%	5.4%	5.8%
	5	97.7%	1.5%	1.5%
	25	105.9%	5.0%	4.8%
	100	107.3%	2.1%	2.1%
	500	98.2%	0.5%	0.5%
nornicotine	1	97.9%	4.0%	3.7%
	5	104.9%	1.5%	1.6%
	25	102.2%	1.8%	2.0%
	100	105.9%	0.7%	1.2%
	500	98.7%	2.0%	1.9%

Table 14. Assay validation parameters for nicotine and metabolites (n = 3).

Therefore, direct quantification of target analytes in urine with this UHPLC-MS/MS method was suitable over the assay range (1-500 ng/mL for nicotine, cotinine, *trans*-3-hydroxycotinine, anatabine

and nornicotine, 2.5-100 ng/mL for nicotine-*N'*-oxide and cotinine-*N*-oxide and 1-250 ng/mL for anabasine).

Eventually, a LOD standing around 0.25 ng/mL could be estimated for nicotine, cotinine, *trans*-3-hydroxycotinine, anabasine, anatabine and nornicotine, and around 0.5 ng/mL for nicotine-*N'*-oxide and cotinine-*N*-oxide.

Selectivity

Selectivity evaluation towards interfering endogenous matrix compounds was conducted on 6 different urine specimens from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days. According to triplicate sample treatment followed by UHPLC-MS/MS analysis, no interfering endogenous molecules were highlighted within selected transition windows. Indeed, MCX provided very clean sample extracts.

Likewise, potential influence of exogenous xenobiotics was assessed relying on the analysis of all the clinical study samples at t_0, accounting for 42 urine specimens. Noteworthy, presence of stimulants commonly found in sport and society was also investigated, in particular caffeine and pseudoephedrine. Accordingly, no exogenous xenobiotic was highlighted and interfered with target analytes within selected transition windows.

Carry-over

Assessment of carry-over was performed by injecting a blank urine sample following the analysis of the highest calibrator for each compound of interest. This experiment was repeated three successive times and no target analyte was detected, confirming the absence of carry-over effect.

Matrix effect

Comparison of signals detected in urine and in the neat solution highlighted concentration-dependent ion enhancement or suppression effects depending on the compound of interest. Indeed, evaluation of ME indicated slight ion suppression or enhancement occurring at low, medium and high concentrations, ranging from 89 to 102%, 93 to 99% and 95 to 101%, due to endogenous matrix compounds competing with target analytes towards ionization (data not shown). According to the excellent repeatability of these measurements (RSD < 10%), the increased sensitivity and selectivity of the method to quantify all substances at LLOQ, the sample treatment method proved fit-for-purpose. Noteworthy, ME was efficiently corrected by the deuterated standards at all concentration levels. Therefore, influence of ME on the quantitative analyses was found negligible.

Stability

Analyte stability was evaluated after investigation on the influence of the routine analytical throughput on compounds of interest in QC samples (*n* = 3) at low, medium and high concentrations. Since stability experiments were designed to mimic actual storage and analysis conditions, QCs were preserved at -20 °C in a complete dark environment followed by a series of 3 successive freeze and thaw cycles within a week, a fourth cycle after three months, a fifth after six months and a final one after one year to assess long-term stability. Likewise, QC samples were left on the bench top at room temperature (21 °C) for 24 h and in the autosampler at 10 ° C for 24h to assess short-term temperature stability.

According to the minimal variations observed in the concentration measurements across the stability tests (RSD < 15%), storage conditions adopted during this study were adequate to ensure a high stability of all compounds of interest.

E. Application to clinical study samples

During the course of the clinical study, a total of 320 urine samples from 21 volunteers were collected. Out of this initial testing pool, urinary measurements from three subjects were excluded, either for canceling the scheduled ST consumption phase on their part or for exhibiting positive concentration values at t_0 despite the 72h nicotine consumption abstinence period required prior to each phase of the study. Therefore, analytical results reported in this document correspond to a population of 18 volunteers, accounting for 288 urine samples.

Sample preparation and quantification were performed according to the methodology described in the experimental and assay validation parts. Also, a quantitative value of 0 ng/mL was assigned to compounds of interest either detected in the LOD to LLOQ concentration range or not detected at all.

Concentration values collected during the quantitative assays allowed building concentration profiles over time to depict the metabolism of nicotine, cotinine, *trans*-3-hydroxycotinine and their related glucuronides. Conversely, concentration distributions of minor nicotine metabolites and tobacco alkaloids could not be exploited due to trace levels being detected at best. Indeed, as the single dose of smoke or ST was not sufficient for measuring these compounds above the LLOQ at almost all collection times, concentration profiles could not be built for the population of volunteers.

Comparing concentrations levels would be misleading as the nicotine dose administered to each volunteer was directly depending on the gesture for both forms of consumptions. In addition, MoNIC badges indicated that self-induced second-hand exposure to smoke, and thus to nicotine, varied from one volunteer to another despite exposure in a controlled environment with a high ventilation rate. Therefore, results should be interpreted with regards to the metabolism rate of each

metabolite or ratios between metabolites rather than concentration values. Reporting urinary biomarkers as normalized ratios to urinary creatinine concentrations to control for variations in urine flow rate did not affect the concentration patterns. As volunteers were encouraged to drink a lot of water on exposition days in order to keep hydrated and ease the blood and urine collection process, the influence of urine concentration was found negligible. Therefore, normalization with creatinine was not relevant in this study and raw data will be presented. On the whole, a very close fit was observed between the urinary profiles obtained after smoke and ST consumption (Figure 22).

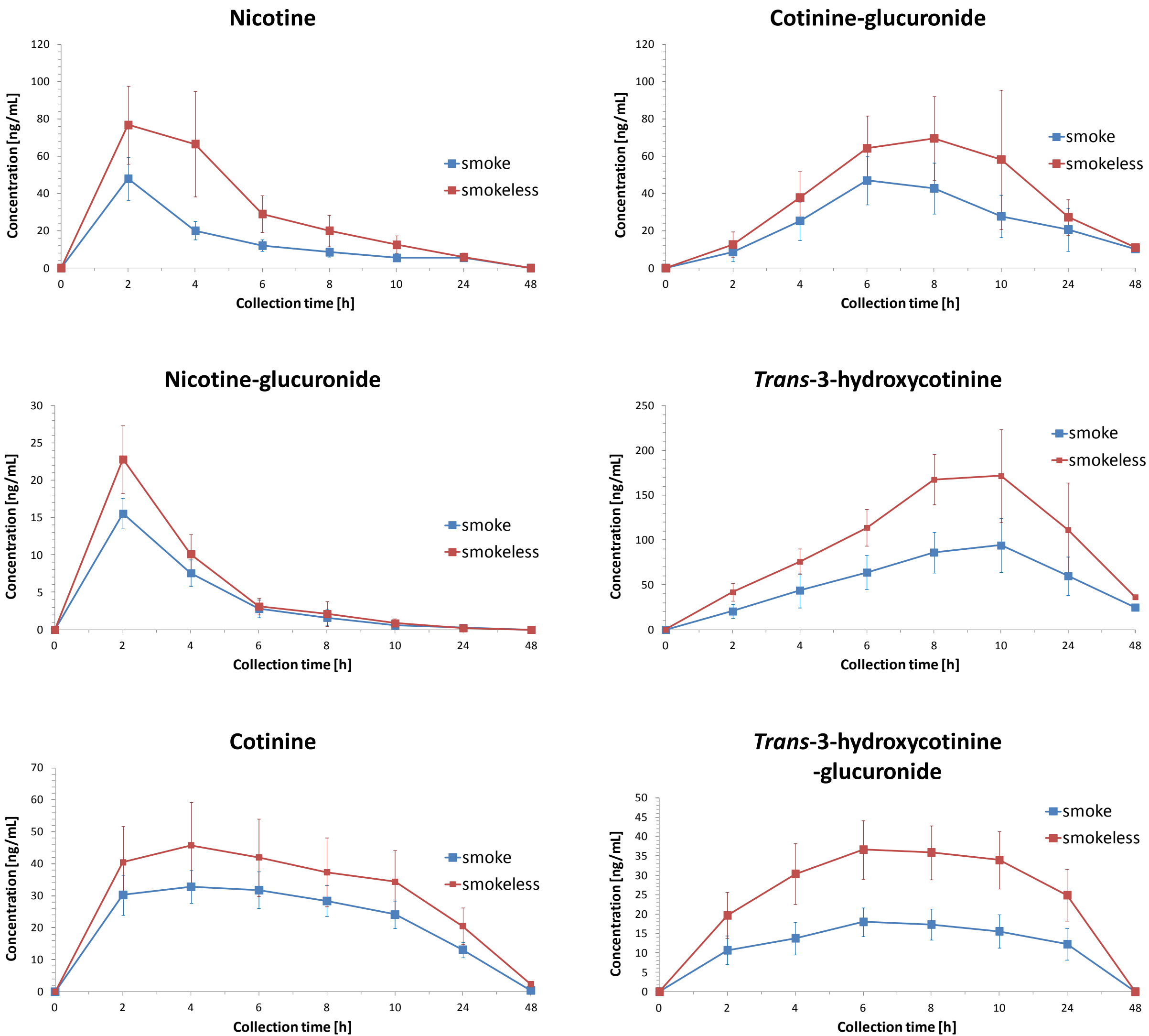


Figure 22. Mean urinary concentration profiles of nicotine and related major phase I and II metabolites after smoke and ST consumption for the 18 volunteers. The vertical lines indicate the standard deviation at 95% confidence intervals.

No cross-over points were observed when comparing smoke and smokeless concentration profiles of all target compounds. Regardless of the product, the highest concentration was measured at t1 (2h) for nicotine and nicotine-glucuronide, t2 (4h) for cotinine, between t3 and t4 (6-8h) for cotinine-glucuronide, at t5 (10h) for *trans*-3-hydroxicotinine and at t3 (6h) for *trans*-3-hydroxicotinine-glucuronide. Therefore, a slight delay was found for cotinine-glucuronide between

cigarette smoking and snus consumption as the highest point was reached at 6h and 8h, respectively. Likewise, while peak concentration of nicotine was reached after 2h for both forms of consumption, the decline of nicotine concentrations in urine appeared to occur at a slightly slower rate with ST (Figure 22 and 23, Table 14).

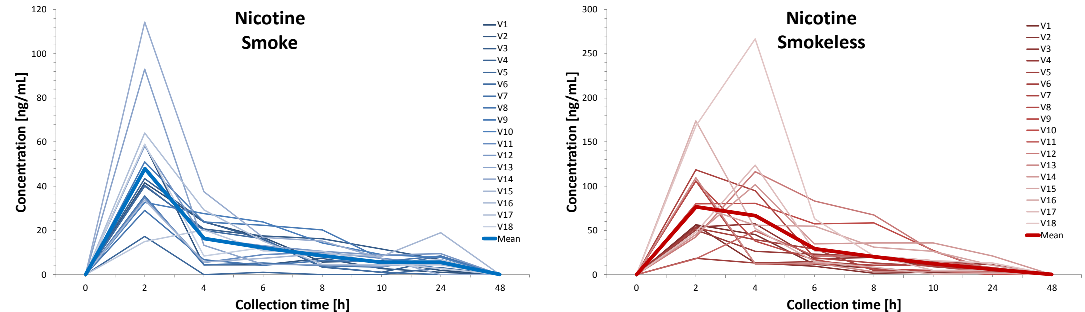


Figure 23. Urinary concentration profiles of nicotine after smoke or ST consumption for the 18 volunteers. The bold lines indicate the mean pharmacokinetic profiles for the entire population of subjects.

Nevertheless, a statistical significance would be difficult to assume due to the limited intervariability between urinary profiles and as concentrations achieved within the 2h intervals prior and after each time point are unknown. Indeed, determining the exact time and true value of peak concentrations would require considerably shorter time intervals between urine collection points, which would be quite inconceivable from a practical point of view. Also, smokeless tobacco consumption showed a higher intra-variability of nicotine concentrations ranges at each time point compared to smoked tobacco (Table 14). A similar situation was observed for the other metabolites (data not shown). This further illustrates the difficulty to highlight a statistically significant difference between urinary profiles after each type of tobacco use.

Product type		Nicotine concentration (ng/mL)							
		t0	t1	t2	t3	t4	t5	t6	t7
smoke	Range	0	23-176	0-58	6-37	0-31	0-18	0-29	0
	Mean	0	74	25	19	13	8	8	0
smokeless	Range	0	27-268	19-410	15-128	2-104	0-55	0-32	0
	Mean	0	118	102	45	31	19	9	0

Table 14. Ranges of urinary concentrations for nicotine at the different time points depending on the type of product consumed by the 18 volunteers.

These observations confirm data previously reported in the literature, indicating a slightly slower absorption rate through the buccal mucosa than the lungs, which may also affect the glucuro-conjugation rate as a consequence (92). However, differences of such limited magnitude would not be sufficient factors for unequivocal distinction between smoke and ST use. With a single exposure to each form of tobacco consumption, metabolic intra-variability for each volunteer remains difficult to ascertain. Therefore, careful assumptions are made here when interpreting differences observed

in the fitting of the concentration profiles. From an anti-doping perspective, the situation is even more complicated and blurry as real life use of snus in sports such as ice hockey induces cumulative effects. Indeed, the community has reported the use of multiple pouches during the games, or at least regular daily use during training. In this context, complexity of the metabolic profiles would certainly be augmented.

Likewise, combining pairs of compounds to build concentration profiles on the basis of metabolites ratios was not found to bring additional relevant information as this data processing tended to further reduce the intra- and inter-variability for the most part. Therefore, urinary profiles of nicotine and related major phase I and II metabolites after smoking or ST use were not found to bring relevant information in the perspective of distinguishing both forms of consumption. According to the literature, concentration in blood is thought to raise at a slower rate with smokeless tobacco, as compared to tobacco smoke (92). Therefore, measuring concentration profiles for blood specimens might be more promising. Similar results might be highlighted though and glucuro-conjugated metabolites will be absent in blood. The additional analysis of combustion products seems to hold more potential. Nevertheless, considering the difficulties to measure minor nicotine metabolites and tobacco alkaloids, one may argue that investing efforts in achieving part 2 of the clinical study may not be worth it. Indeed, as pyrolysis products are found in low levels in regular cigarette smokers, the single dose administered in this study might result in undetectable trace amounts or low concentrations compatible with environmental exposure. Practically, the profiles indicate that analyzing for nicotine and major phase I and II metabolites in urine can be considered as a screening procedure only. DS routine analyses should integrate nicotine and major phase I metabolites to highlight nicotine consumption. Then, a confirmatory analysis would be necessary to distinguish between smoke and smokeless tobacco use. As discussed in this section, such a method has not been developed yet and would require to complete part 2 of the clinical study. In these views, the compatibility with daily activities of Accredited Laboratories is seriously limited. Indeed, due to the significant prevalence of nicotine reported during the two previous studies, further analysis of suspicious samples with a confirmation method based on the analysis of combustion products would probably bring too much analytical pressure to fit with routine anti-doping analyses.

On a further note, the recent birth of the electronic cigarette (e-cigarette) trend in Western countries is prone to complexity the situation, both from bioanalytical and behavioral perspectives. As an electronic vaping device delivering nicotine, the e-cigarette does not involve pyrolysis and associated combustion products but shares the same route of absorption as tobacco smoke. From a bioanalytical point of view, concentration profiles for part 1 and 2 obtained after e-cigarette use might be similar to tobacco smoke. Nevertheless, no controlled study has been reported in the literature yet and the metabolism of nicotine for this type of consumption remains unknown. In

addition, the composition of e-cigarette fluids available on the market is generally not properly labeled (170). Indeed, levels of nicotine, tobacco alkaloids, TSNA, aldehydes, metals, flavors, etc. vary considerably between and within brands (171). Likewise, the delivery and release of these compounds are not consistent. Eventually, nicotine levels indicated on e-cigarette fluids often significantly differs from the measured content. Considering the chemical heterogeneity of e-cigarette cartridges and refill solutions might come up as an alibi provided by the athlete to disguise snus-like concentrations profiles. Since e-cigarette is an ever-increasing societal phenomenon, this excuse would also significantly challenge the assumption of ST use for performance-enhancement if a case was brought to court.

F. Conclusion

Completion of part 1 of the clinical study allowed building urinary concentration profiles of nicotine, cotinine, *trans*-3-hydroxycotinine and related glucuronides for 18 volunteers after smoke and ST consumption. Metabolism of nicotine-*N'*-oxide, cotinine-*N*-oxide, anabasine, anatabine and nornicotine could not be plotted due to single dose exposures resulting in trace amounts of these minor compounds.

A very close fit was highlighted when comparing urinary concentration profiles obtained for cigarette and snus consumption. Despite a seemingly slower metabolic rate for nicotine excretion and a slight delay in reaching peak concentration for cotinine-glucuronide after snus consumption, results did not bring promising information in the perspective of distinguishing smoke from ST use. The additional analysis of pyrolysis products throughout part 2 of the study would most likely be very valuable from a theoretical point of view. However, from a practical perspective, as difficulties were experienced in measuring trace amounts of minor nicotine metabolites, the ability to quantify combustion products is very uncertain. Investing extensive efforts in the development of an analytical method would not be reasonable. Indeed, even if overcoming this analytical challenge were to be successful, the resulting methodology would certainly be of confirmatory nature, thus relying on a nicotine screening procedure at first. Considering the steady analytical workflow of Accredited Laboratories and the significant prevalence of nicotine in numerous sports disciplines, multiplying and developing more complicated analytical tools would be somewhat irrational.

In light of all these elements, the ability of analytical tools to answer the phenomenon is questionable and another angle of approach is required to address ST use in sport. The limitations and challenges faced in this study put additional emphasis on the need for tackling the problem from the public health perspective. A preventive rather than repressive attitude should be adopted in order to propose proactive solutions to defuse emerging addiction and health problems associated with tobacco consumption.

ST offers undoubtedly numerous advantages due to its odorless nature, ease of sharing, using and dissimulating during sports practice and at a school. Given the tobacco-free policies established at most schools in Western countries, ST may facilitate nicotine dosing among young (113). On a further note, perception of high ST use among peers and teammates might also be an initiation factor. With elite athletes acting as role model to lower level athletes, alleged or real improvement of performance in sport might be an additional driving force for use. The risks of this alternative to cigarette smoking are amplified by the relatively high nicotine content which is likely to initiate strong addiction issues. A contentious debate exists over the prediction of snus use as a potential gateway to smoking onset (172-174). Despite prospective studies in the United States arguing in this direction, a recent research found no evidence for an increased risk of smoking among ST users (175). Whether gateway theory applies to ST or not, the use of snus in the sports environment needs actions to promote cessation and discourage initiation, particularly among the youth.

Previous studies have found intervention through peer-led educational sessions and cessation counseling as part of quit strategies in American high schools very efficient in helping consumers to stop using ST (113). However, preventing initiation through these strategies has proven to be a difficult goal to achieve. In views of the American experience, tackling this problematic is difficult and the anti-doping world would have to work hand in hand with the sports community, educational system and public health to refine existing strategies and develop new ones targeting populations at risk, among which the youth is a priority. Promoting health is a mission stated in the Code and anti-doping bodies have to pursue educational and preventive ambitions. In order for the message to be well-received, helping cessation and preventing initiation is a more positive and promising answer than including nicotine to the Prohibited List in a repressive attitude. Nevertheless, prohibition has helped to reduce prevalence of other social drugs like cannabinoids.

On a side note, the investigator has been contacted by the European Healthy Stadia Network (HS) organization and the World Heart Federation in the summer of 2013, rising concerns about ST consumption spreading throughout stadia. As health professionals, HS is keen to see a ban on the sale, promotion and use of all tobacco products within stadia and sports environments, to the benefit of fans, athletes and those living in local communities near to sports stadia. Whilst the argument for banning the sale and use of smoking tobacco in stadia is now well substantiated, banning all forms of tobacco is more difficult for stadia and governing bodies to buy into. Much of this can be put down to the promotional and sponsorship activities of tobacco companies who are now targeting sports clubs through major sponsorship and advertising deals, and hence building major resistance to a ban on ST. The situation of the e-cigarette flooding the market is an additional concern as health issues have not been fully investigated yet and as quality control of the production process is not well regulated either (176, 177).

CHAPTER IV | CONCLUSION & PERSPECTIVES

IV.1. Conclusion & perspectives

Anti-doping finds itself at a crossroads where the constant refinement of doping methods is becoming a reality requiring a change of paradigm in the lead of the fight. Research in anti-doping has been focusing almost exclusively on testing in order to meet the technological requirements linked to the continuous evolution of the List. Nevertheless, while bioanalytical tools for detecting the abuse of prohibited substances rapidly improved within the last decade, the problem simultaneously evolved towards more professionalized individual and organized doping. The phenomenon today is global and involves not only athletes, but also a wide variety of actors from physicians possessing state-of-the-art knowledge on how to dope with minimal risks to get caught to entourage, and a complex chain of supply for prohibited substances. The 2012 USADA vs. Lance Armstrong case is probably the most striking example of highly sophisticated doping where no adverse analytical finding was reported over the course of the most extraordinary road cycling career in history. Traditional doping with high doses of pharmaceutical preparations has been replaced by modern methods relying on micro doses and an extensive catalogue of pharmaceutical and/or designer drugs. This strategy has proven quite efficient in achieving the desired physiological response while challenging the capacity of Accredited Laboratories to detect and quantify prohibited agents in biological samples.

Regardless of the individual or organized character of doping, this practice may involve mechanisms of criminal nature in the production and distribution of prohibited substances. Operating with methods borrowed from criminal organizations, doping networks have drastically increased in reach and density. From the ground level up to clandestine pharmaceutical plants, the supply of prohibited substances is a very profitable business. This attractiveness translates into growing numbers of producers and trafficking routes, hence the difficulty to disrupt doping when solely focusing on the identification of cheating athletes. A parallel may be drawn with the problem of illicit drugs trafficking where dismantling production and distribution networks has shown more impact than strictly repressing users prone to addiction issues. As athletes are end-products of a business, identifying and punishing these individuals has only little leverage and deterrent effect on doping networks. Accredited Laboratories unarguably possess state of the art technology serving very sophisticated research and development programs. Nevertheless, targeting the most likely cheaters for collecting biological samples remains a challenge. Indeed, statistics on AAFs remain quite stable despite a tremendous increase of anti-doping tests and bioanalytical sensitivity and accuracy.

Despite testing remaining indispensable to effective anti-doping programs, exploring new scientific avenues to understand and highlight doping offenses so as to exert efficient preventive or

disruptive effects would be a welcomed addition. In this context, learning from Forensic Intelligence to develop Anti-Doping Intelligence might be an innovative approach to address the different aspects of doping, from the individual level up to the organized doping and trafficking level in a proactive rather than reactive way. Through logical processing of multiple sources of information gathered in a structured memory, Anti-Doping Intelligence might be invaluable for detecting and describing potential, emerging or existing doping trends. In turn, anti-doping authorities and partners of the fight would be provided with timely, accurate and usable information for decision-making to solve, reduce and/or prevent this phenomenon. Depending on the level of intelligence, focus would either be put on providing investigative leads on athletes and/or individuals responsible for the diffusion of doping, or on organized doping and the trafficking of doping to agents to identify their mechanisms of action and disrupt them, or eventually on the bigger picture to understand and prevent predisposing factors of doping.

Forensic Intelligence being a wide area of research requiring field evaluation and validation for practical implementation, the studies on the use of ST in sport were a first attempt at illustrating phenomenon detection and follow-up functions. Starting with collating into a structured memory epidemiological studies on snus consumption in society, pharmacological studies on the potential of nicotine for performance-enhancement and information within ice hockey clubs on widespread snus use, a strong interest arose in investigating on the phenomenon. A prevalence study on nicotine consumption was first conducted during the 2009 IIHF World Championships as evidence pointed at this sport discipline in the first place. Analytical chemistry tools were developed and validated to highlight the use of nicotine by athletes. According to the results of the study, 53% of the ice hockey players consumed nicotine close to or/and during the games. As this prevalence was alarming, the premises of an intelligence-driven approach of the phenomenon were initiated to gain knowledge on the extent and seriousness of the problem and to provide a better description and understanding of current consumption patterns. Measuring the prevalence over a longer period of time and across a large number of sports appeared particularly relevant. Accordingly, a one-year monitoring study of nicotine use was conducted over 2010-2011 across all the IC samples received at LAD. Again, the results indicated a very significant prevalence among winter sports (up to 43.8% in snowboarding), as well as several ball sports (up to 55.6% in American Football) and individual disciplines (up to 37.5% in gymnastics). Following this study, WADA added nicotine to the 2012 Monitoring Program, which was a significant step towards the acquisition and further integration of global data to an organized memory. Through longitudinal monitoring of IC samples by seven different Accredited Laboratories around the world, WADA could obtain a large-scale picture of nicotine consumption distribution over time and space.

Due to the need for being able to distinguish between smoke and ST consumption and between recreational and doping purpose, the project was further pursued. A clinical study was designed and conducted in order to develop analytical and biostatistical tools for discriminating one form of consumption from another. During the first part of the study, the concentration profiles of nicotine metabolism and minor tobacco alkaloids were drawn by analyzing urine specimens after cigarette, and alternatively snus, consumption by healthy male volunteers. Since the route of administration differs, differences in the metabolic rate could be reasonably expected. The idea was to provide a better understanding of nicotine metabolism after smoke and ST consumption. Completion of this part of the study allowed building urinary concentration profiles of the most abundant nicotine metabolites. The high degree of similarities observed when comparing these profiles highlighted the need for more complex analytical tools to distinguish smoke from ST use. In particular, the study of combustion products, as only one consumption form involves pyrolysis, would be very valuable. From an anti-doping perspective, the bioanalytical methodology would have to fit with routine analyses while offering a satisfactory discriminative power. However, compatibility with routine screening procedures and the analytical workflow of Accredited Laboratories was a prime necessity that could be predicted as nearly impossible to fulfill. In these views, investing further human, temporal and economical resources into the study of pyrolysis products was not found reasonable.

Despite being an unresolved yet, the challenge faced in distinguishing tobacco consumption forms is informative to WADA for decision-making on ST in sport. Indeed, these difficulties and the scientific data obtained through the prevalence and clinical studies stress the need to support educative and preventive programs on ST consumption rather than testing and repression. With respects to the health risks associated with tobacco and amplified by addiction issues due to nicotine, strategies of intervention should focus on proposing educational material, peer support and counseling and medical follow-up. Prevention programs should be designed to be well-received by athletes and the sport environment, but also by the sports community at large, from schools to stadia, comprising all categories and ages of sports enthusiasts. In a future perspective, this thesis work could be pursued in this direction, by developing, implementing and assessing the impact of tailor-fitted strategies for ST cessation and prevention of initiation in sport. As the e-cigarette is gaining huge popularity, researching on these aspects would be particularly relevant.

Additional data would also be required to complete the logical process and to obtain a truly intelligence-led approach. In these regards, a behavioral study has been designed based on the randomized-response survey model. Every ice hockey player in a Swiss National or Regional League, as well as every junior ice hockey player, will be asked to fill in an extensive questionnaire addressing nicotine consumption. The project will be conducted in collaboration with the Institute of Sport

Sciences of UNIL (ISSUL) of which Prof. Fabien Ohl will be the lead supervisor of the study, the tobaccology department of the PMU and the Institut Universitaire de Médecine Sociale et Préventive (IUMSP), both affiliated to CHUV. Collating global data collected with the Monitoring Program since 2012 and behavioral information on the perception and aim of ST consumption by the athletes would be very valuable. Indeed, this type of data would help determining the purpose of nicotine use, whether as a recreational practice or as a performance enhancer, and to understand the decision process behind engaging in this practice. Also, these randomized-response surveys will provide interesting prevalence data. When integrated to the structured memory fed by the research works mentioned previously, this study would add a great value to the general picture of the phenomenon.

The intelligence-inspired approach followed in this thesis on ST use in sport provides anti-doping authorities and the sports community with comprehensive information on a little-studied phenomenon. The logical reasoning process was conducted to help with decision-making of proactive and problem-solving nature to positively impact on ST through the support of educational programs and peer-led assistance. From a public health perspective, prevention of initiation, risk reduction and help in cessation seek to provide long-term solutions. Considering the public health dimension of the fight against doping, this attitude would be a novel and interesting approach to detect and tackle emerging or existing consumption dangers.

Anti-Doping Intelligence possesses an unarguable potential for the gathering of information on any type of phenomenon in order to understand its underlying mechanisms and to identify key points for intervention. Following the research initiated in this thesis, further evaluating and putting to test all the different aspects of the concept in the real world would be essential to come up with an operational system. If indeed such a system could be placed in a central location (WADA perhaps) and supplied and supported by anti-doping professionals, there is no doubt that this would be a major step forward.

CHAPTER V | APPENDICES

V.1. List of Publications

1. Time for change: a roadmap to guide the implementation of the World Anti-Doping Code 2015. Dvorak J., Baume N., Botré F., Broséus J., Budgett R., O Frey W., Geyer H., Harcourt P., Ho D., Howman D., Isola V., Lundby C., Marclay F., Peytavin A., Pipe A., Pitsiladis Y., Reichel C., Robinson N., Rodchenkov G., Saugy M., Sayegh S., Segura J., Thevis M., Vernec A., Viret M., Vouillamoz M., Zorzoli M. Br J Sports Med 2014 April; 48, 801-806.
2. Harcourt P., Marclay F., Clothier B. A forensic perspective of the AFL investigation into peptides: An anti-doping investigation case study. BJSM 2014 March; 48, 810-813.
3. Marclay F., Jan N., Esseiva P., Mangin P., Margot P., Saugy M. Le changement de paradigme du Renseignement Forensique pour la lutte contre le dopage organisé et le traffic de substances interdites. RICPTS 2013 Dec; 4 (13), 451-72.
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6. Bélanger R., Marclay F., Berchtold A., Akre C., Saugy M., Suris JC. To what extent does adding tobacco to cannabis expose young users to nicotine? Nicotine Tob Res 2013 Feb; 15 (11), 1832-8.
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9. Marclay F., Saudan C., Vienne J., Tafti M., Saugy M. Source inference of exogenous gamma-hydroxybutyric acid (GHB) administered to humans by means of carbon isotopic ratio analysis: novel perspectives regarding forensic investigation and intelligence issues. Anal Bioanal Chem 2011 Apr; 400 (4): 1105-1112. *Published as Paper in Forefront*
10. Marclay F., Saugy M. Determination of nicotine and nicotine metabolites in urine by hydrophilic interaction chromatography-tandem mass spectrometry: Potential use of smokeless tobacco products by ice hockey players. J Chromatogr A 2010 Nov; 1217(48):7528-38.
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V.2. Original Papers

V.2.1. Marclay F., Jan N., Esseiva P., Mangin P., Margot P., Saugy M. 2013

Le changement de paradigme du Renseignement Forensique pour la lutte contre le dopage organisé et le trafic de substances interdites.

The paradigm shift of Forensic Intelligence for the fight against organized doping and the trafficking of prohibited substances.

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Résumé

Bien que la lutte contre le dopage poursuive de nombreux objectifs à fin de détection, réduction, neutralisation et/ou prévention du dopage, l'approche actuelle se focalise essentiellement sur le processus judiciaire. A l'instar de la prise de décision dans le domaine de l'application de la loi, l'antidopage pourrait voir la portée et l'efficacité de son activité s'améliorer sensiblement en s'appuyant sur le Renseignement Forensique. En effet, des bénéfices notables pourraient découler d'une récolte d'information à plus large spectre, suivie d'une structuration et d'un traitement des données approfondis. Le Renseignement Forensique pourrait apporter une dimension logique étendue à l'interprétation des données relatives aux activités de dopages afin de supporter une approche globale et proactive outrepassant les limitations de l'approche actuelle réactive et du cas par cas. L'information provenant d'une variété de sources telles que des études physiologiques, épidémiologiques et sociologiques alimenterait une mémoire organisée afin de fournir du renseignement en temps réel sur la taille, la gravité et l'évolution de tout phénomène relatif au dopage. Du fait de la complexité du dopage, intégrer ces éléments aux résultats de chimie analytique des athlètes et au suivi longitudinal des biomarqueurs de dopage serait d'une grande aide pour élaborer des stratégies adaptées à chaque situation, que le phénomène soit global ou un cas isolé. Le processus du renseignement se fonde avant tout sur un raisonnement logique. La polyvalence et flexibilité en découlant seraient de précieux atouts pour détecter, neutraliser, mettre fin ou/et prévenir le dopage, individuel ou organisé, ou le trafic de produits dopants. Cette méthodologie permettrait également d'affiner le ciblage des athlètes ou équipes et de fournir des preuves de dopage en l'absence de résultats d'analyses anormaux. Ainsi, par le Renseignement Antidopage une réponse proactive pourrait être proposée à tout phénomène de dopage potentiel ou émergent, ou aux problèmes existants en présentant des actions ou/et politiques innovantes.

Summary

Despite pursuing numerous goals in the detection, reduction, solving and/or prevention of doping, today's approach of Anti-Doping is mostly centered on the judicial process. Similarly to decision-making in the area of law enforcement, Anti-Doping might significantly improve its reach and efficiency by relying on Forensic Intelligence. Indeed, considerable benefits might result from a more extensive gathering of knowledge followed by an in depth

structuring and processing of the data. Forensic Intelligence might bring a broader logical dimension to the interpretation of data related to doping activities to support a more global and proactive approach to overcome the limitations of the current reactive and case-based process. Information coming from a variety of sources, such as physiological, epidemiological and sociological studies, would feed an organized memory to provide real time intelligence on the size, seriousness and evolution of the phenomenon. Due to the complexity of doping, integrating these elements to analytical chemical results of the athletes and longitudinal monitoring of doping biomarkers would help in designing strategies adapted to each situation, whether the phenomenon is global or an isolated case. The intelligence process is based on the elaboration of a logical reasoning in the first place. The resulting versatility and flexibility would be precious assets for detecting, neutralizing, disrupting or/and preventing doping, whether individual or organized, and the trafficking of doping agents. This methodology would also allow refining the targeting of athletes or teams and to bring evidence on doping in absence of adverse analytical findings. Therefore, through Anti-Doping Intelligence a more proactive response might be proposed to any potential or emerging phenomenon, or to existing problems by presenting innovative actions or/and policies.

Mots clefs: dopage dans le sport; dopage organisé; trafic de drogue; renseignement forensique.

Keywords: doping in sport; organized doping; drugs trafficking ; forensic intelligence.

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1. Introduction

En réponse aux scandales de dopage sur le Tour de France durant les années 1990 et en particulier l'affaire Festina de 1998, l'Agence Mondiale Antidopage (AMA) fut fondée en 1999 (Müller 2010). En 2004, la première édition du Code Mondial Antidopage (le Code) fut acceptée internationalement afin de réguler et uniformiser la lutte contre le dopage à travers le monde (World Anti-Doping Agency (WADA) 2004). Suite à une première révision en 2009, une seconde édition est entrée en vigueur. (World Anti-Doping Agency (WADA) 2009). Le programme antidopage mondial, tel qu'établi par le Code, vise à préserver la valeur intrinsèque du sport en garantissant le droit fondamental de tout athlète à participer à un sport exempt de dopage et en promouvant la santé, l'éthique, l'honnêteté et l'égalité. Ce texte assure également l'harmonisation et la coordination des programmes antidopage à l'échelle nationale et internationale afin de détecter, neutraliser et prévenir le dopage. En parallèle, le Comité Liste de l'AMA constitué d'un panel d'experts est chargé de maintenir et proposer une mise-à-jour annuelle de la Liste des Substances et Méthodes Interdites (la Liste) ainsi que du Programme de Surveillance spécifiant les substances autorisées pour lesquelles la prévalence d'utilisation peut être mesurée à l'aide des échantillons récoltés pour les contrôles antidopage (World Anti-Doping Agency (WADA) 2013; World Anti-Doping Agency (WADA) 2013). Bien que le dopage puisse concerner l'ensemble de la population sportive, il incombe uniquement aux athlètes de haut niveau licenciés à une Fédération Sportive signataire du Code d'accepter ses règles comme condition de participation à une manifestation sportive s'inscrivant dans le calendrier de cette même Fédération.

Afin de répondre aux besoins scientifiques liés à l'évolution constante de la Liste, la recherche dans l'antidopage se focalise avant tout sur l'analyse des produits interdits dans les fluides biologiques. Par conséquence, le domaine accumule les outils et méthodes analytiques plutôt que de proposer de nouvelles perspectives et un regard fondamental sur le phénomène. En effet, les Laboratoires Accrédités par l'Agence Mondiale Antidopage (AMA) se doivent de consacrer l'essentiel de leurs ressources au développement de méthodes plus sensibles et sélectives employant une technologie moderne et sophistiquée. A l'inverse, la communauté ne prête qu'une attention périphérique à la compréhension et appréhension du phénomène en tant que tel en s'appuyant sur des données scientifiques objectives et des sources d'information alternatives. En ce sens, il serait intéressant d'élargir les perspectives de lutte en incluant un aspect de renseignement. A ce sujet, la situation du dopage et de la criminalité traditionnelle

partagent nombre de similarités de par les mécanismes qui les régissent et le contexte légal dans lequel elles s'insèrent. Le Renseignement Forensique ayant amené des solutions fort intéressantes ces dernières années afin de lutter contre la criminalité et/ou dans une optique sécuritaire, il serait opportun d'explorer cette voie dans le contexte de l'antidopage. Dès lors, ce papier propose de discuter la problématique actuelle du dopage puis d'évaluer la plus-value que pourrait apporter le Renseignement Forensique après l'avoir présenté.

2. La lutte contre le dopage

2.1. Approche et défis actuels

La conduite actuelle de la lutte contre le dopage est axée quasi exclusivement sur le processus judiciaire traditionnel dont elle est l'instrument essentiel et souffre d'un manque de liberté pour s'en détacher (Figure 1). Celui-ci est composé de trois phases successives : le problème à identifier et trouver, la collecte des indices de preuve pour prouver le problème, et finalement le procès (Ribaux, Baylon et al. 2010).

Figure 1. Les trois phases du processus judiciaire traditionnel dans l'antidopage.

La fiabilité et l'intégrité du système judiciaire sont assurées par un système qualité couvrant l'ensemble des étapes liées à la pratique de la lutte, allant de la collecte d'échantillon jusqu'à la présentation des résultats d'analyse anormaux devant le Tribunal Arbitral du Sport (TAS). Néanmoins, bien que ce processus s'améliore constamment, l'efficacité de la lutte contre le dopage devrait être mesurée selon les progrès accomplis dans la détection, réduction, neutralisation et/ou prévention du dopage et non pas seulement en termes de statistiques sur le nombre de cas positifs. En effet, ces concepts sont autant de buts poursuivis par le Code et servant à la protection de la santé (World Anti-Doping Agency (WADA) 2009).

En se basant sur le nombre de profils sanguins anormaux, la Fédération Internationale d'Athlétisme (IIAF) a pu estimer à 14% la prévalence du dopage sanguin sur l'ensemble des échantillons prélevés entre 2001 et 2009 chez les sportifs d'élite participant aux compétitions d'athlétisme (Sottas, Robinson et al. 2011; Zorzoli 2011). En particulier, la prévalence selon le pays d'origine de l'athlète variait chez l'homme et la femme de 3 à 48% et de 1 à 46%,

respectivement. De même, une prévalence allant de 10 à 35% a été avancée dans un sondage sur le dopage réalisé auprès des athlètes d'élite Allemands en 2008 (Pitsch and Emrich 2012). Malgré tout, moins de 2% des ~250'000 échantillons sanguins et urinaires collectés chaque année signalent l'usage d'une substance ou méthode interdite (Zorzoli 2011; World Anti-Doping Agency (WADA) 2012). Ce chiffre restant stable en dépit de l'énorme croissance du nombre de tests et de la sensibilité et fiabilité des méthodes analytiques.

Cela s'explique en partie par le perpétuel perfectionnement des méthodes de dopage afin de ne pas être détectées par les méthodes de chimie analytique des laboratoires, un temps de latence existant entre l'apparition de nouveaux médicaments aux propriétés dopantes sur le marché et leur inclusion à la Liste et la nature aléatoire des contrôles réalisés hors-compétition. Bien que les méthodes de dopage les plus sophistiquées soient avant tout l'apanage des sportif d'élite, le dopage par l'usage de nouvelles substances dopantes non-réglementées touche également les athlètes de toute catégorie.

En effet, ces dernières années, la pratique du dopage a évolué vers le micro-dosage continu et le mélange d'un cocktail de drogues de synthèse de sorte à atteindre l'augmentation de performance désirée (Howman 2012). A l'inverse des prises ponctuelles d'une substance unique en fortes doses, cette méthodologie offre l'avantage d'aboutir à des niveaux de concentrations quasi indétectables. Comme illustré par les cas Marion Jones et Lance Armstrong en 2007 et 2012, les athlètes jouissant d'un suivi médical permettant le dopage intelligent peuvent ne pas être détectés tout au long de leur carrière malgré le nombre colossal de contrôles subis (United States Anti-Doping Agency (USADA) 2007; United States Anti-Doping Agency (USADA) 2012). L'introduction des contrôles hors-compétition au début des années 1990 dû à l'abus massif de stéroïdes anabolisants androgènes durant les périodes d'entraînement à travers les années 1980 fut un changement de paradigme énorme pour la lutte contre le dopage (Saugy, Robinson et al. 2009). Néanmoins, bien que complémentaires aux contrôles en-compétition et une amélioration majeure afin de dissuader la pratique du dopage, l'efficacité de ces tests se heurte à des défis organisationnels, économiques et scientifiques. Contrôler chaque athlète de façon régulière serait complètement utopique et démesuré compte tenu des coûts, du nombre d'échantillons à analyser et des conflits avec la vie privée des athlètes ainsi générés. Dans ce contexte, affiner le ciblage des athlètes est nécessaire, mais le perfectionnement grandissant du dopage rend difficile la mise-en-évidence des tricheurs potentiels et de tester ces individus à temps avant que les agents dopants ne

soient totalement éliminés de l'organisme. Dès lors, et à l'exception du Passeport Biologique de l'Athlète aidant le ciblage du dopage sanguin, les contrôles hors-compétition souffrent toujours d'un facteur aléatoire. Ainsi, seule la pointe de l'iceberg ne semble être perçue avec la pratique actuelle de l'antidopage.

A ce sujet, l'usage, ou la tentative d'usage, par un athlète d'une substance ou méthode prohibée ne constitue que l'une des huit violations de règle décrites dans le Code Mondial Antidopage de 2009 (World Anti-Doping Agency (WADA) 2009). La possession et le trafic de substances interdites, au même titre que l'administration à un athlète en compétition d'une substances ou méthode prohibée, sont également interdits. Toutefois, seul un faible effort de recherche a été consacré à ces violations de règles, ne conduisant qu'à l'élaboration d'approches scientifiques succinctes à caractère exploratoire restreint. A l'image de la portée globale du texte de loi tel que considéré dans son ensemble, la lutte contre le dopage se doit de multiplier les perspectives selon lesquelles elle aborde une problématique aussi complexe. En effet, sachant qu'un nombre croissant d'études indiquent l'existence de réseaux de dopage organisé et d'un marché gris/noir pour le trafic de substances interdites, ces aspects méritent approfondissement et l'élaboration de stratégies adaptées (Waddington 2000; Donati and World Anti-Doping Agency (WADA) 2007; Jan, Marclay et al. 2011; Lentillon-Kaestner 2013). L'affaire BALCO (Bay Area Laboratory Co-Operative) qui se déroula à San Francisco (USA) en 2003, l'Opération Puerto de 2006 en Espagne et le cas Lance Armstrong de 2012 illustrent particulièrement ces questions. A ce jour, les investigations conduites durant ces affaires demeurent plutôt uniques en leur genre, indépendamment de l'ampleur du phénomène qu'elles révélèrent.

Dans une certaine mesure, la situation actuelle résulte d'une répartition des ressources humaines et temporelles devant répondre avant tout aux défis et besoins scientifiques, légaux et organisationnels de la lutte. Au quotidien, les Laboratoires Accrédités sont absorbés par un flux constant et important d'échantillons sanguins et urinaires dont les analyses sont commanditées par les Organisations Antidopage Nationales et Régionales, les Fédérations Sportives Nationales et Internationales et le Comité International Olympique (CIO), ainsi que par l'expertise scientifique demandée pour les cas suspicieux. A cela s'ajoutent l'activité de recherche, notamment le développement et la validation de méthodes pour analyser les nouvelles substances et méthodes identifiées, les programmes de tests d'aptitude analytique, les audits concernant la gestion de la qualité, l'évaluation régulière du respect des normes ISO

17025 et les exigences spécifiques des Standards Internationaux pour les Laboratoires (World Anti-Doping Agency (WADA) 2012). Ces différents aspects constituent naturellement une priorité pour les Laboratoires Accrédités dans l'accomplissement de la mission qui leur est conférée par les autorités antidopage.

David Howman, le directeur de l'AMA depuis 2003, déclara à ce propos (Howman 2012):

« Tester est – et a toujours été – le fondement de la lutte contre le dopage dans le sport ; la science étant utilisée contre la science, avec l'espoir que "notre" science – celle de la communauté antidopage – devienne un jour trop sophistiquée pour que les athlètes risquent de se doper ».

Cette déclaration illustre le fait que les Laboratoires Accrédités portent sur leurs épaules la majeure partie de la lutte contre le dopage, avec l'espoir de régler définitivement la problématique par le progrès dans les méthodes de dépistage.

Ce cadre de travail favorise une évolution technologique effrénée afin de détecter, identifier et quantifier chaque substance apparaissant sur la liste, et garantit la validité des résultats analytiques ainsi qu'un suivi complet de la chaîne de possession. Toutefois, du fait des aspects opérationnels décrits précédemment, les chercheurs et autres partenaires de la lutte sont contraints de concentrer leurs réflexions essentiellement sur le cadre du processus judiciaire traditionnel. En conséquence de cette approche au cas par cas orientée justice, une vision stratégique pour comprendre le phénomène et proposer des solutions à long terme fait encore défaut à l'antidopage. Les ressources actuelles pour colliger de l'information pertinente afin de prévenir l'apparition de phénomènes, de démanteler le dopage organisé et le trafic de substances prohibées sont restreintes et le domaine focalise ses efforts sur la résolution de cas isolés. Dès lors, il serait intéressant d'élargir les perspectives de lutte en incluant un aspect de renseignement. Cette approche sera discutée par la suite en tissant un parallèle avec les problématiques forensiques connexes à l'antidopage bénéficiant de sa plus-value.

2.2. Cas de dopage récents

L'affaire BALCO illustra pour la première fois le rôle décisif que peuvent jouer les investigations policières à fin de démanteler un cas de dopage organisé. A l'origine de l'investigation fédérale américaine qui débuta en 2003, une source anonyme fournit au

Laboratoire Olympique Accrédité de l'Université de Californie à Los Angeles (UCLA) une seringue contenant de la tétrahydrogestrinone (THG), un stéroïde anabolisant androgène de synthèse alors inconnu des autorités antidopage (Catlin, Sekera et al. 2004). Par la suite, Trevor Graham, un coach de sprint dont les clients incluaient Marion Jones et Tim Montgomery, fut identifié comme étant l'informateur anonyme. Lorsque le laboratoire détenu par Victor Conte fut perquisitionné par la U.S. Internal Revenue Service Criminal Investigations Unit et la San Mateo County Narcotics Task Force en 2003, de larges quantités d'agents dopants de toutes sortes et du matériel relatif aux activités du laboratoire furent saisis (Kazlauskas 2010). Selon les archives, BALCO était responsable pour la conception et la distribution du THG. Les investigations révélèrent que de nombreux athlètes de très haut niveau étaient listés dans les documents saisis, notamment Marion Jones (trois fois médaillée d'or aux Jeux Olympiques et deux fois médaillée d'argent), Tim Montgomery (recordman du monde du 100m), Barry Bonds (recordman du nombre de homeruns en une saison de baseball) et Kelli White (championne du monde du 100 et 200m) (USA Today 2007).

Cette investigation fut couronnée de succès puisqu'elle aboutit à la condamnation de nombreux athlètes pour dopage malgré l'absence de résultats d'analyse anormaux. A plus d'un titre, cet événement sans précédent fut particulièrement marquant dans l'histoire de la lutte contre le dopage (Kazlauskas 2010). Une conséquence intéressante de cette collaboration fructueuse entre l'Agence Américaine Antidopage (USADA) et les différents services d'investigation cités précédemment fut la reconnaissance de sources d'information alternatives comme moyen de preuve fiable pour identifier et prouver l'existence d'un cas de dopage. Cette mention fut d'ailleurs ajoutée à la révision du Code en 2004 (Bowers 2012). Ce scandale éveilla également la conscience populaire sur l'utilisation très répandue d'agents dopants et des limitations de la législation antidopage dans les sports professionnels Américains, conduisant rapidement à l'amélioration des programmes de contrôles antidopage aux Etats-Unis avec le soutien de la Maison Blanche.

De même, en 2006, l'Opération Puerto fit éclater au grand jour l'immense réseau de dopage organisé du Dr. Fuentes, l'ancien médecin de l'équipe cycliste Kelme, impliquant dopage sanguin et transfusion, la prise de stéroïdes et d'hormone de croissance, etc. (Court of Arbitration for Sport (TAS-CAS) 2010; Court of Arbitration for Sport (TAS-CAS) 2012). Le cas fit surface suite aux confessions du cycliste Jesús Manzano sur le dopage systématique opéré par le Dr. Fuentes auquel il prit part durant ses années sous sa supervision médicale.

Comme le dopage apparaît dans le code pénal espagnol, la Guardia Civil conduit l'investigation et saisit 200 poches de sang et 105 agents dopant de toute sorte (testostérone, l'érythropoïétine humaine recombinante, hormone de croissance, insuline, etc.) lors de la perquisition du bureau et du domicile du Dr. Fuentes. De plus, un calendrier d'entraînement et de dopage contenant une liste codée d'une centaine de noms fut découvert, laissant apparaître une majorité de cyclistes de haut niveau et indiquant de fortes suspicions de dopage sanguin pour de nombreux joueurs de football et de tennis. En recoupant ces informations avec le suivi hématologique des cyclistes professionnels, quelques athlètes ayant gagné les courses cyclistes les plus prestigieuses (Tour de France, Vuelta, Giro, Pro Tour, Olympic Games, etc.) purent être identifiés et convaincus de dopage. Alors que la collaboration entre les autorités sportives et la police espagnole fut fructueuse et prometteuse, leurs efforts conjoints se heurtèrent à la réluctance des politiques quant à la poursuite des investigations afin de dévoiler l'ensemble des noms apparaissant sur la liste.

En 2012, l'affaire United States Antidoping Agency (USADA) vs. Lance Armstrong démontra à nouveau le rôle inestimable des investigations criminelles afin de détecter un dopage organisé hautement professionnalisé. Malgré le manque de preuves bioanalytiques directes, l'investigation de l'USADA permis de récolter les témoignages sous serment de 26 personnes, incluant 15 cyclistes connaissant les activités de dopage de l'équipe US Postal Service Pro Cycling Team, ainsi que de 11 coéquipiers de Lance Armstrong. A ce propos, 9 de ces coéquipiers étaient eux-aussi des clients du Dr. Michele Ferrari supervisant le dopage organisé au sein de cette formation cycliste. Les preuves récoltées dans cette affaire couvrent l'ensemble de la carrière de Lance Armstrong depuis 1998 et sont de types documentaires, scientifiques, directs et/ou circonstanciels. En effet, l'investigation permis de collecter une multitude de documents bancaires et comptables attestant du versement de plus d'un million de dollars au Dr. Ferrari durant leur collaboration et une communication par courrier électronique abondante durant une période pour laquelle Lance Armstrong prétendait n'avoir eu aucune relation professionnelle avec son médecin. A cela s'ajoutent encore de vastes quantités de données additionnelles incluant les analyses sanguines du coureur durant sa carrière et l'expertise scientifique réalisée a posteriori afin d'interpréter les résultats selon une logique longitudinale. L'ensemble de ces éléments permis de démontrer de manière univoque l'utilisation, la possession et la distribution de substances augmentant la performance par Lance Armstrong (United States Anti-Doping Agency (USADA) 2012; United States Anti-

Doping Agency (USADA) 2012). Selon l'USADA, *« l'équipe US Postal Service Pro Cycling Team conduisait le programme de dopage le plus sophistiqué, professionnalisé et couronné de succès que le sport ait jamais vu. »* (United States Anti-Doping Agency (USADA) 2012). Sous ce régime, Lance Armstrong établi le record sans précédent de gagner le Tour de France sept fois consécutivement entre 1999 et 2005 tout en n'étant jamais contrôlé positif.

Ces exemples illustrent une utilisation tactique de l'information afin de fournir des pistes d'investigation et d'alimenter le processus judiciaire. Néanmoins, ces cas auraient difficilement vu le jour sans confessions ou dénonciations. Dès lors, une utilisation plus systématique et approfondie des données paraît nécessaire afin de favoriser une détection et une identification proactive du dopage individuel et organisé. Afin d'exercer un véritable effet dissuasif sur les athlètes, la dimension temporelle est cruciale. En effet, l'impression que seuls les malchanceux sont attrapés subsiste naturellement lorsqu'une violation de règle n'est découverte que plusieurs années après que l'athlète ait profité de la célébrité et de la gloire associées à ses victoires.

3. Le Renseignement Forensique

3.1. Concept général

Les sciences forensiques sont un aspect important du système judiciaire, que ce soit à but investigatif ou pour fournir un support scientifique à des questions légales. L'analyse peut être fondée sur la logique plutôt que les outils.

Le Renseignement Forensique adopte cette approche et poursuit un objectif supplémentaire dans l'étude des activités criminelles en apportant une dimension logique plus vaste pour l'interprétation des traces détectées et collectée suite à une activité litigieuse. Les caractéristiques de ces vestiges sont extraites et décrites avant d'être intégrée à une mémoire structurée contenant de l'information préalablement acquise sur le phénomène. En procédant à une analyse logique, des inférences peuvent être construites, permettant de révéler un réseau de liens hypothétiques entre les informations fraîchement acquises et celles mémorisées précédemment. Le raisonnement est entièrement basé sur le postulat et l'évaluation d'hypothèses émises sur la relation entre les éléments organisés au sein de la mémoire structurée. L'analyse est donc une étape interprétative où chaque nouvel élément peut confirmer les liens présumés et/ou connecter des informations initialement considérées

comme non-apparentées. A l'inverse, ce processus logique peut remettre en question, voire exclure, l'existence des liens supposés précédemment.

L'information véhiculée par les traces peut être organisée dans une mémoire à court terme pour exploitation directe dans une affaire criminelle, tout comme dans une mémoire à long terme pour décrire et suivre les tendances et/ou séries criminelles ainsi qu'identifier les vulnérabilités du système. De par une exploitation minutieuse des traces afin de fournir de l'information sur le phénomène criminel étudié, le renseignement peut ainsi soutenir la prise de décision de nature tactique, opérationnelle ou stratégique dans le domaine de l'application de la loi pour résoudre, réduire et/ou prévenir les crimes.

En pratique, le renseignement est constitué de trois niveaux de fonction, à savoir le *renseignement tactique*, *opérationnel* et *stratégique* (Figure 2).

Figure 2. Organisation et cibles de chaque niveau de Renseignement Forensique. De l'approche réactive de micro-niveau du renseignement tactique à la planification de la réduction du crime à moyen-niveau du renseignement opérationnel et à l'approche proactive à niveau global du renseignement stratégique.

Ces différents niveaux de renseignement opèrent sur une échelle spatiale et temporelle croissante. Toutefois, partageant une logique de raisonnement autour de la trace et bon nombre de sources d'information, ces concepts liés au Renseignement Forensique interagissent plutôt que d'être des procédés distincts. De ce fait, la délimitation entre ces différents niveaux doit être perçue comme souple de part leur recouvrement naturel.

Le *renseignement tactique* est une approche réactive supportant la prise de décision des agents de police en temps réel et proposant des pistes pour la conduite des investigations sur des cas spécifiques. Le processus d'analyse logique des traces fournit de l'information exacte, actuelle et utilisable pour la détection, l'identification, la localisation et l'arrestation de

délinquants potentiels, et pour la collecte des preuves à fin de poursuite judiciaire (Figure 3) (Ribaux, Walsh et al. 2006). Le *renseignement tactique* seule ne cherche pas à mettre en évidence les activités criminelles existant à l'échelle dépassant celle de l'individu ou constituant des problèmes répétitifs (Ratcliffe 2007). La détection de tels phénomènes criminels et leur neutralisation, réduction ou prévention requiert des sources d'information plus larges et une exploitation plus en profondeur de la trace comparée à l'approche à court terme et au cas par cas de le *renseignement tactique*.

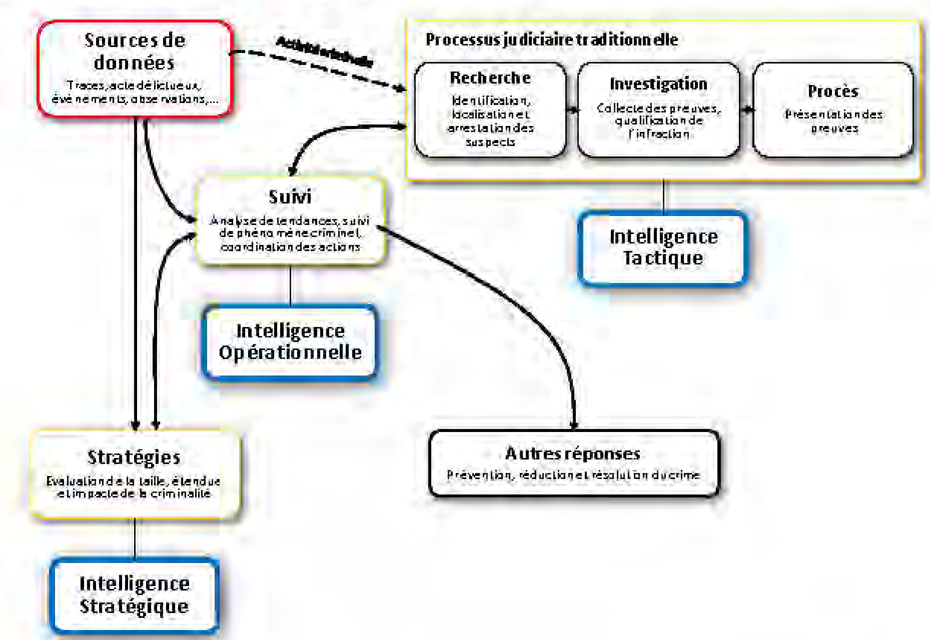

Figure 3. Structure du Renseignement Forensique au travers du traitement logique de l'information supportant le renseignement tactique alimentant le processus judicaire, le renseignement opérationnel pour planifier des actions de réduction du crime et le renseignement stratégique pour développer des solutions à long terme orientées vers le futur (Ribaux and Margot 2007).

Le *renseignement opérationnelle* demande un niveau d'organisation plus élevé pour fournir une compréhension étendue des tendances criminelles, en assurer le suivi et aider à la coordination des actions (Figure 3) (Ratcliffe 2007). Ce concept cherche à frapper les problèmes à répétition tels que la criminalité sérielle, l'activité des organisations criminelles ou le trafic de stupéfiants en promouvant une résolution du problème par une vision plus

proactive et orientée vers le moyen terme. En effet, le *renseignement opérationnel* assiste les preneurs de décisions dans le ciblage et le déploiement de ressources policières et judiciaires ainsi que dans la planification d'actions pour la réduction et la prévention du crime (Ribaux, Girod et al. 2003).

A l'aide de méthodes exploratoires, statistiques et de visualisation relatives à l'analyse criminelle, d'importantes quantités d'informations sauvées dans une mémoire structurée sont traitées logiquement pour détecter des problèmes géographiques et/ou temporels, pour déterminer le type de délinquant et identifier des modes opératoires criminels, aussi appelés *modus operandi*. Comme ce processus analytique est itératif dû au flux constant de nouvelle information remplissant la mémoire, le *renseignement opérationnel* supporte le renseignement criminel de sécurité avec de la connaissance constamment affinée et mise-à-jour.

Enfin, le *renseignement stratégique* opère à un niveau d'organisation plus globale. La criminalité est un phénomène complexe évoluant constamment à travers le temps et l'espace dû à des changements démographiques, économiques, politiques, environnementaux, etc. Dès lors, le *renseignement stratégique* prend en compte ces paramètres d'une perspective criminologique ainsi que les sous-niveaux du Renseignement Forensique. Le raisonnement est celui d'une approche multivariée visant à la description et la compréhension des mécanismes criminels au sein d'un environnement en perpétuel changement (Figure 3) (Ratcliffe 2007). Le *renseignement stratégique* est orientée vers le futur et résolument proactive avec l'intention de pressentir l'apparition de menaces criminelles potentielles ou émergeantes en tentant d'identifiant et résoudre les vulnérabilités du système de manière préventive. Une conséquence intéressante de le *renseignement stratégique* est la capacité d'identifier les domaines pour lesquels le maintien de l'ordre et les actions de minimisation du risque pourraient aboutir à un résultat positif (Morelato, Beavis et al. 2013).

Cette approche cherche à frapper le phénomène dans son ensemble plutôt que sur des activités criminelles spécifiques. Le *renseignement stratégique* peut contribuer à éclairer les choix des stratégies les plus efficaces pour contenir les problèmes identifiés. Ce concept est propice à proposer des solutions à long terme ainsi que des programmes ou actions préventives et éducatives. De par la nature des buts poursuivis, cette approche peut également prendre une dimension politique.

3.2. Perspectives pour l'Antidopage

3.2.1. Transposition du concept

Le concept du Renseignement Forensique décrit précédemment pourrait être transposé en Renseignement Antidopage en s'appuyant sur une méthodologie similaire et en changeant le paradigme pour celui du dopage dans le sport (Figure 4).

Figure 4. Transposition du concept du Renseignement Forensique au Renseignement Antidopage.

Au niveau tactique, là où le Renseignement Forensique se focalisait sur les individus, le Renseignement Antidopage se concentrerait sur les athlètes. L'exploitation des traces, comprenant notamment résultats bioanalytiques, documents liés à la pratique du dopage et à la distribution, administration et/ou consommation de substances prohibées, ou encore saisies de produits interdits, s'inscrirait dans la perspective de proposer des pistes d'investigation sur des athlètes et/ou individus impliqués dans la diffusion du dopage, et d'apporter un support scientifique cohérent au processus judiciaire.

De même, au niveau opérationnel, l'attention passerait de la criminalité sérielle et de l'activité des organisations criminelles aux tendances dans l'abus de produits prohibés, au dopage organisé et au trafic de substances interdites. La sérialité pourrait se définir ici comme le caractère récurrent de ces pratiques dopantes ou liées au dopage. Parmi elles, le dopage sanguin à l'érythropoïétine humaine recombinante (rEPO) dont l'usage a débuté dans les années 1990 et connaît des vagues d'utilisation successives à chaque mise sur le marché d'une nouvelle génération du médicament. La continuité et l'étendue de l'abus de stéroïdes anabolisants (SAAs) depuis les années 1980 ainsi que leur trafic à l'échelle planétaire illustrent également une forme de sérialité. A titre d'exemple, le dopage d'Etat en Europe de

l'Est était organisé systématiquement par les gouvernements dans leur quête de reconnaissance internationale par opposition à leurs performances économiques et scientifiques plutôt inférieures (Klaus Müller 2010). Bien que cette pratique fût caractéristique de tous les pays satellites de l'Union soviétique, la République démocratique allemande (Allemagne de l'Est) a également utilisé cette stratégie afin d'être reconnu comme Etat à part entière après la division de l'Allemagne par les Alliés à la fin de la Seconde Guerre Mondiale. Les résultats incroyables obtenus dans les sports de haut niveau par ce pays relativement petit furent acclamées dans le monde entier, créant une image forte malgré la politique répressive et l'infériorité économique de ce régime. Les AAs et autres produits de synthèse développés dans des laboratoires de recherche secrets afin d'alimenter le dopage systématique sont en grande partie responsables de ces performances sportives hors du commun et de l'entretien du mythe pendant des décennies. Il en va de même à l'heure actuelle avec les médecins du sport supervisant des programmes de dopage organisé, allant de l'import, voire même la fabrication, des produits à leur administration et au contrôle des valeurs physiologiques des athlètes afin de demeurer indétectable. L'exploitation des traces servirait à détecter ces problèmes, à en mesurer les dimensions temporelles et géographiques et en identifier le *modus operandi*. Etoffer la connaissance sur la pratique du dopage ou en lien avec cette activité aboutirait à une meilleure compréhension aussi bien de la structure du phénomène que de son principe de fonctionnement. A ce sujet, l'analyse de scénario est propice à l'étude des formes complexes de crime et utile à l'élaboration de stratégies de lutte plus efficaces (Chiu, Leclerc et al. 2011). Ce concept considère le crime sous l'angle du *modus operandi* plutôt que comme un événement unique. Dès lors, l'analyse de scénario vise à mapper la séquence complète d'actions et de décisions avant, pendant et après la commission du crime afin d'identifier les points clés du mode opératoire et de proposer des mesures propres à les contrer. En révélant les points faibles potentiels où le processus de commission du crime pourrait être dérangé ou interrompu, voire prévenu, cette méthodologie va permettre de réduire les opportunités et les ressources tout en augmentant les risques liés à la perpétration du crime. L'analyse de scénario pouvant être rétrospective, étudier les grandes affaires de dopage organisé précédemment citées sous cet angle serait d'une grande valeur afin de comprendre les mécanismes régissant cette forme de dopage et d'agir en conséquence.

Enfin, au niveau stratégique, l'accent serait porté sur la compréhension du dopage dans son ensemble plutôt que sur celle de la criminalité. Le raisonnement chercherait à identifier

les facteurs propices au développement du dopage au sein de la communauté sportive, qu'ils soient humains, environnementaux, économiques, ou même politiques. Avec une compréhension étendue des mécanismes du dopage, la dimension stratégique du renseignement permettrait idéalement de prévenir le phénomène en proposant des solutions visant à minimiser, voire neutraliser, l'influence des facteurs identifiés.

Etant donné l'écart considérable entre l'estimation de la prévalence du dopage relevée dans différents sondages et les statistiques annuelles de la WADA sur les résultats d'analyse anormaux, l'occasion devrait être saisie d'exploiter plus en détail les informations à disposition et véhiculées par les traces pour rendre la lutte plus efficace (Sottas, Robinson et al. 2011; Zorzoli 2011; Pitsch and Emrich 2012; World Anti-Doping Agency (WADA) 2012). Afin d'illustrer le concept du Renseignement Antidopage, les cas du dopage organisé et du trafic de substances interdites seront discutés dans le paragraphe suivant.

3.2.2. Dopage organisé et trafic de produits dopants

A. Tour d'horizon

Avec plus de 200 substances apparaissant sur la Liste des Substances et Méthodes Interdites de 2013, le trafic de produits dopants est un commerce attractif et lucratif dont la dimension est probablement sous-estimée et qui emprunte des chemins complexes relevant du crime organisé (Donati and World Anti-Doping Agency (WADA) 2007; World Anti-Doping Agency (WADA) 2013).

Les sources d'approvisionnement peuvent varier, en particulier selon le type et le statut légal d'une substance. En effet, certaines préparations pharmaceutiques contenant des agents dopant comme la pseudoéphédrine ne requièrent pas de prescription et peuvent être achetées directement en pharmacie. A l'inverse, les médicaments à usage thérapeutique tels que la rEPO ou l'hormone humaine de croissance (HGH) nécessitent une prescription ou alternativement peuvent être contrefaits par des laboratoires clandestins ou détournés des stocks de production de l'industrie pharmaceutique et proposés sur le marché gris (Kazlauskas 2010). De ce fait, il existe un trafic d'agents dopants aussi bien authentiques que contrefaits. A cela s'ajoutent un certain nombre de substances illicites vendues par des trafiquants de stupéfiants sur le marché noir, telles que la cocaïne ou les amphétamines, et également considérées comme des agents dopants.

Par conséquent, le dopage s'avère être un phénomène complexe en considérant la variété de substances acheminées par voie légale et illégale et l'étendue des connections entre les individus impliqués dans ces réseaux de distribution. Alors que le dopage pourrait apparaître comme étant la pratique d'un athlète de façon isolée, elle implique toujours une ou plusieurs entités dans la fourniture en produits dopants ou dans leur utilisation, que ces personnes soient des amis ou membres de la famille, ou l'équipe médicale, le directeur ou les coéquipiers d'une équipe sportive, ou des chimistes, biologistes et pharmaciens, ou des industries pharmaceutiques et laboratoires clandestins, ou des organisations criminelles et trafiquants de drogues (Figure 5).

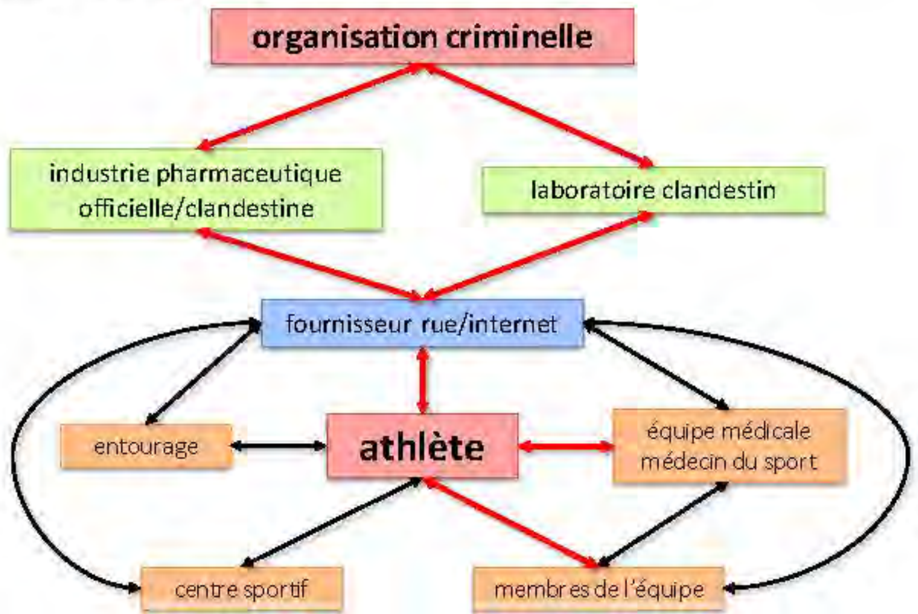

Figure 5. Réseau de dopage et diversité des liens potentiels entre les différentes entités pouvant le constituer

Du fait de l'absence de régulation quant à la production et le commerce d'agents dopants dans la plupart des pays, il existe des terreaux fertiles pour le développement d'usines pharmaceutiques clandestines. Dans l'ensemble, les industries en Thaïlande, Chine, Inde et Russie jouent un rôle majeur avec de nombreux sites de production et des routes de transite bien établies (Donati and World Anti-Doping Agency (WADA) 2007). Les statistiques sur les saisies de produits dopants pointent la Chine et l'Inde comme sources d'approvisionnement subissant la plus forte croissance sur le marché gris international. Les chiffres sont d'ailleurs

corroborés par l'expansion constante de leurs industries pharmaceutiques. A l'échelle planétaire, la production en Thaïlande, Chine, Inde, Russie, Grèce et au Mexique représente environ 55% de la distribution globale. Un indice de l'implication de l'industrie dans ce commerce se trouve dans la surproduction de la rEPO estimée à cinq ou six fois supérieure aux besoins thérapeutiques mondiaux. En effet, un éventail d'experts soutenus par des institutions gouvernementales ont relevé le déséquilibre significatif entre la production pharmaceutique de médicaments aux propriétés dopantes, en particulier la rEPO, HGH et testostérone, et la demande thérapeutique (Donati and World Anti-Doping Agency (WADA) 2007).

Selon les statistiques, la production globale des SAAs est estimée à environ 700 tonnes par année, ce qui suffirait à fournir 15 millions de personnes par année. En particulier, environ 70 tonnes de testostérone sont produites pour satisfaire les besoins de 1.5 millions de personnes par année. De même, la production de rEPO et HGH atteint environ 34 millions de fioles par année, ce qui suffirait à traiter environ 2 millions de personnes (Donati and World Anti-Doping Agency (WADA) 2007). La diffusion du dopage, qui touche approximativement 15.5 millions de personnes, ne concerne pas que les athlètes de haut niveau, mais différentes catégories d'individus. Selon le rapport Donati, le dopage dans son ensemble est représenté par 35-37% d'athlètes de tous niveaux, 38-40% de bodybuilders et d'amateurs de fitness, incluant des agents de surveillance privés, 4-6% de militaire et de forces de police, 1-2% de personnes dans l'industrie du spectacle et 15-20% de faux traitements (Donati and World Anti-Doping Agency (WADA) 2007). Selon une étude récente menée aux Pays-Bas, environ 8.2% des membres de centres de fitness avouent utiliser des substances de tous types augmentant la performance (Stubbe, Chorus et al. 2013).

L'internet a amené de nouveaux horizons au trafic de produits dopants en simplifiant et sécurisant son développement global. L'absence de régulation sur la production de ces substances dans la plupart des pays et la multiplication des "pharmacies" en ligne assurent la facilité et sécurité de ce marché gris qui est en constante augmentation. En effet, il y a sensiblement moins de risques pour les producteurs et trafiquants de laisser les stocks de production dans le pays d'origine et de les envoyer par poste en quantités réduites plutôt que de charger des camions de stocks importants de produits et effectuer un voyage à travers plusieurs frontières jusqu'à destination finale.

Différentes formes de trafic ont été identifiées (Donati and World Anti-Doping Agency (WADA) 2007). La méthode traditionnelle consiste à charger de larges quantités sur un moyen de transport jusqu'à des destinations intermédiaires, où une partie du chargement est livré, alors que le reste est acheminé jusqu'à la prochaine destination. Cette forme était la plus populaire avant l'apparition du commerce en ligne et devient de moins en moins fréquente de nos jours. Une méthode plus commune, et similaire à la forme traditionnelle, se base sur le service postal pour la distribution finale des produits après transport jusqu'aux destinations intermédiaires. L'avantage est de pouvoir rassembler différents produits dopants dans un endroit spécifique afin de mettre en place une "pharmacie" en ligne qui enverra de petits paquets aux acheteurs. Enfin, la méthode la plus simple et subissant la plus forte croissance implique de stocker les agents dopants directement sur le site de production et de les envoyer par paquet après commande en ligne.

Même si l'approvisionnement en substances pourrait paraître se situer sur l'échelle individuelle de l'athlète, ses risques majeurs résident dans le dopage organisé et le marché largement contrôlé par les organisations criminelles. La mafia italo-américaine a contrôlé le trafic de SAAs et HGH aux Etats-Unis jusqu'au milieu des années 1990 et à l'émergence de la criminalité organisée russe comme acteur dominant du marché gris. Suite à ces événements, la part de l'Asie dans la production et le trafic mondial de produits dopant s'est considérablement étendue au point de remplacer la mafia russe comme leader du marché gris. Dans de nombreux cas, les mêmes organisations criminelles ont été identifiées comme responsables aussi bien du trafic de stupéfiants et de substances interdites que de celui des médicaments contrefaits (Dégardin, Roggo et al. 2011).

B. Renseignement et contexte légal

Malgré la conscience des autorités sur l'existence du phénomène depuis plusieurs décennies, lier les différentes entités constituant un réseau de dopage demeure une pratique difficile et inexplorée, et ce particulièrement du fait que seuls quelques pays reconnaissent le dopage comme une infraction pénale. En effet, le dopage est une affaire de droit pénal publique en Espagne, Italie et Belgique, ce qui définit un cadre légal aux actions relatives à la justice criminelle et notamment l'utilisation des investigations policières (McKenzie 2007). Alternativement, des pays comme les Etats-Unis et l'Australie définissent le dopage dans le

sport comme une affaire de droit privé. Dès lors, les demandes quant à une violation de règle se discutent entre la Fédération Sportive Internationale et l'athlète avant d'être transmises à l'Organisation Nationale Antidopage, œuvrant comme organe administratif, ou à une cours d'arbitrage privée, en particulier le Tribunal Arbitral du Sport. A l'inverse, la situation en France depuis la révision de la législation antidopage en 2006 est celle d'un compromis entre des pouvoirs administratifs renforcés et la criminalisation d'activités spécifiques liées au dopage des athlètes (République Française 2006). Par conséquent, l'angle sous lequel le phénomène est abordé reflète les différences de statuts légaux propres à chaque pays. D'un côté les processus administratifs se focalisent généralement sur la poursuite des athlètes ayant violé une loi antidopage, alors que l'utilisation de mécanismes de justice criminelle permet des investigations additionnelles visant le dopage organisé et d'agir sur les différents acteurs impliqués dans le dopage d'un athlète.

A l'exception de ces quelques juridictions, les organisations criminelles profitent d'une relative sécurité due au manque de collaboration, et de bases légales paralysant le partage d'information pertinente détenue à différents niveaux par différents acteurs comme l'AMA, les Organisations Antidopage Nationales et Régionales (NADO et RADO), les Fédérations Sportives Nationales et Internationales, le CIO, les Laboratoires Accrédités, les agences nationales de contrôle des frontières, l'Organisation Mondiale des Douanes (WCO), les services de police nationaux, l'Office de Police Européenne (EUROPOL) et l'Organisation de Police Criminelle Internationale (INTERPOL). En effet, ces entités sont autant de sources d'information, provenant soit de leur contact direct avec les athlètes, équipes sportives ou médecins du sport, des études de prévalence des laboratoires, des saisies douanières de produits dopants, ou encore des investigations policières sur des activités criminelles dont le dopage peut faire partie. Une fois ces informations collectées et structurées, leur traitement selon les principes de l'Analyse Criminelle pourrait être utile et particulièrement efficace de sorte à identifier le dopage systématique ou les réseaux de trafic et de neutraliser, mettre fin à et/ou prévenir ces activités (Ribaux, Walsh et al. 2006). Alors que de l'information pertinente est entre les mains des autorités antidopage et de leurs partenaires, l'absence généralisée de réglementation est un obstacle majeur à l'implémentation du Renseignement Antidopage. En considérant le dopage comme un problème de santé publique de part la dangerosité des produits consommés et le fait qu'une frange jeune de la population soit concernée laissent espérer une prise de conscience des politiques.

Une approche de Renseignement Forensique a été développée à l'Ecole des Sciences Criminelles (ESC) de l'Université de Lausanne, Suisse, afin d'aider à la compréhension et à la lutte face au trafic de stupéfiants ainsi que pour la détection et la description des organisations criminelles impliquées dans la contrefaçon de préparations pharmaceutiques. Les agents dopants englobant une large partie de ces catégories de substances, la méthodologie précitée peut être transposée à l'Antidopage. Dès lors, le terme "produit" sera utilisé sans distinction particulière, qu'il s'agisse de stupéfiants et médicaments ou d'agents dopants.

Alors que la vente en ligne est sujette à une popularité grandissante, le concept de la veille stratégique sur internet peut apporter une information intéressante. En effet, l'utilisation de moteurs de recherche et d'un système d'alertes automatiques par mots clefs permet d'inventorier et suivre les sites de vente en ligne afin d'obtenir un vaste panorama du marché et de détecter l'apparition de nouvelles tendances (Pazos, Giannasi et al. 2013). L'extraction des données numériques sur le codage du site, l'emplacement de la société, l'adresse de contact, etc., peut indiquer la provenance du produit et ses zones de vente à travers le monde, la géolocalisation du revendeur et parfois même son identité. L'analyse de ces traces numériques peut également établir un lien entre sites de vente en ligne apparentés et par conséquent améliorer la compréhension de la structure et de l'activité du marché. Appliquer cette méthodologie à l'analyse des forums, blogs, réseaux sociaux et autres médias en ligne peut sensiblement contribuer à augmenter la connaissance sur l'activité des réseaux de distribution. Ces outils fournissent notamment une vue d'ensemble de l'offre et la demande et permettent d'estimer la prévalence de consommation des substances et d'en suivre l'évolution. A cette information, le profilage physique et chimique des produits saisis apportent une information complémentaire visant à inférer la source de production.

Ainsi, le produit lui-même est une trace du réseau et constitue dès lors un élément direct et d'importance majeure lié à la compréhension de l'organisation criminelle. Il véhicule de l'information fournissant des indications de source et d'activité décisives dans la perspective de décoder la structure du phénomène. Le profilage des stupéfiants par exemple, est vu comme le procédé consistant à extraire des profils physiques et chimiques pour identifier des liens afin d'appréhender l'organisation du trafic de drogues illicites (Morelato, Beavis et al. 2013; Pazos, Giannasi et al. 2013). L'étude des caractéristiques physiques de l'emballage et du produit se focalise sur la description d'une multitude de paramètres, notamment le nom, le logo, la formulation (tablette, gélule, ampoule, crème, etc.), la couleur, les dimensions, les

numéros de lot et/ou de série, le pays de production et la langue. Ces éléments fournissent d'importantes informations liées au producteur et à son *modus operandi*. Suite à l'examen physique, l'analyse chimique va chercher à identifier et mesurer le principe actif et les excipients d'une saisie, permettant la mise en évidence d'un lien entre produits semblables ou entre produits différents provenant d'une même chaine de production (Esseiva, Dujourdy et al. 2003; Been, Roggo et al. 2011).

L'examen physique, la caractérisation chimique, les données numériques et toutes autres informations circonstancielles doivent être stockées dans une mémoire structurée pour une exploitation ultérieure. Ensuite, des modèles d'inférence sont construits sur la base de ces données pour lier des saisies de produits, mettre en évidence des réseaux de distribution et identifier les sources d'approvisionnement. Ces modèles permettront donc d'évaluer l'état du marché gris afin de soutenir la prise de décision et définir les priorités (Esseiva, Ioset et al. 2007). Dans un même temps le profilage chimique permet d'identifier les différentes substances composants la matrice de la saisie et ainsi mettre en évidence d'éventuels risques pour la santé sachant que le principe actif des produits contrefaits présente parfois de grandes différences par rapport aux indications présentes sur les emballages, tant en terme de concentration que de composition (Dégardin, Roggo et al. 2011).

Au niveau de le *renseignement opérationnel*, des profils chimiques et/ou physiques similaires peuvent mettre en lumière un lien entre différentes saisies alors qu'aucune autre information ne le permettait a priori (Morelato, Beavis et al. 2013). Quant au *renseignement stratégique*, elle permet de visualiser l'organisation des réseaux de la production à la distribution de sorte à identifier les points clés ainsi que les tendances dans l'approvisionnement et l'utilisation de ces produits. Il est également possible d'établir des cartographies des principaux pays producteurs de substances interdites ainsi que de la prévalence de consommation de chaque type de produit par zone géographique.

L'extraction du profil physique et chimique des saisies de produit dopants aux douanes et la veille stratégique sur internet fournissent des données qui renseignent sur la vue d'ensemble du marché, permettent d'identifier de nouveaux médicaments à finalité de dopage, et de mesurer l'étendue et la dangerosité d'un épiphénomène de dopage. Etablir des schémas indiquant des relations entre individus et/ou substances pourrait sensiblement améliorer la compréhension de la structure du trafic de substances interdites et par la même améliorer le

taux de succès des interventions visant ces activités. La collection et le partage d'informations sensibles sont essentiels pour évaluer en temps réel, l'évolution, la taille et les mécanismes d'un phénomène de dopage. Toutefois, les saisies douanières ne représentent qu'un faible pourcentage du marché des substances interdites car seule une partie des colis traversant les frontières sont contrôlés et les saisies ne concernent que les produits pour lesquels une législation régulant leur import et/ou consommation est en vigueur. Dès lors, la veille stratégique et le profilage sont complémentaires.

Explorer cette approche serait d'autant plus intéressant dans le cadre de l'antidopage qu'elle pourrait potentiellement aider à un ciblage plus précis des athlètes ou équipes sportives, à l'identification d'individus promouvant la pratique du dopage et au déploiement d'opérations adéquates pour démanteler les ramifications reliées au noyau même des réseaux de dopage. L'avantage global de ce concept consisterait à extraire de l'information d'une nature différente et proposant une plus-value par rapport à celle utilisée actuellement et pouvant ainsi soutenir efficacement les mesures non plus uniquement tactiques, mais également opérationnelles et stratégiques.

4. Conclusion

De par le perpétuel perfectionnement du dopage et sa complexité grandissante, un changement de paradigme s'impose à la lutte telle que conduite aujourd'hui. Alors que la décennie passée fut synonyme d'une évolution rapide des outils technologiques afin de détecter l'abus de substances interdites, cette même période a vu la naïveté des athlètes se dopant dans les années antérieures laisser la place à un dopage individuel ou organisé sensiblement plus sophistiqués. En effet, la réalité est celle d'une épidémie mondiale impliquant des groupes d'athlètes entourés de docteurs sans scrupules à la pointe des techniques de dopage présentant un faible risque de détection. Le cas Lance Armstrong de 2012 est probablement l'exemple le plus flagrant de dopage hautement sophistiqué où aucun résultat d'analyse anormal ne fut reporté au cours de la carrière cycliste la plus extraordinaire de l'histoire. Le dopage traditionnel aux doses massives de quelques produits pharmaceutiques a été remplacé par le dopage moderne aux microdoses d'une multitude de préparations pharmaceutiques et/ou drogues de synthèse. Cette stratégie a démontré son efficacité pour l'obtention de la réponse physiologique désirée tout en défiant la capacité des

Laboratoires Accrédités à détecter et quantifier les agents dopants dans les échantillons biologiques.

Même si le dopage individuel demeure une norme, sa pratique est presque toujours plus ou moins intimement liée à une activité criminelle. Ce constat ainsi que la situation du dopage organisé et le foisonnement des activités criminelles liées à la production et distribution des substances prohibées est un problème majeur pour les autorités antidopage. Opérant selon des méthodes empruntées aux organisations criminelles, voire même directement intégrées à leur activité dans le trafic de stupéfiants, les réseaux de dopage ont drastiquement augmenté en surface et densité. Du niveau de base jusqu'à celui des usines pharmaceutiques clandestines, le dopage est un commerce extrêmement profitable, d'où la difficulté d'interrompre le phénomène dans son entier en se focalisant uniquement sur la détection des athlètes dopés. En tant que produits finaux de ce commerce, identifier et punir ces individus n'a que peu d'effet de levier et dissuasif auprès des réseaux de dopage. A ce sujet, la situation actuelle va probablement prévaloir dû à l'écart significatif entre la prévalence du dopage et les statistiques sur les résultats d'analyses anormaux reportés annuellement. Les Laboratoires Accrédités possèdent sans aucun doute une technologie de pointe au service de programmes de recherche et développement sophistiqués. Toutefois, aussi longtemps que le ciblage des tricheurs pour la collecte des échantillons restera une difficulté de premier ordre, leur travail sera confronté à cette limitation et en souffrira.

Ainsi, suivre le chemin du Renseignement Forensique et développer le Renseignement Antidopage serait une approche innovante afin de traiter des différents aspects du dopage, depuis le niveau individuel jusqu'au niveau du dopage organisé et du trafic de substances d'une manière proactive plus que réactive. Par le traitement logique de multiples sources d'information rassemblées dans une mémoire structurée, la valeur du Renseignement Antidopage serait inestimable pour détecter et décrire des tendances de dopage potentielles, émergentes ou existantes. A ce titre, les autorités antidopage ainsi que les partenaires de la lutte recevraient en temps réel de l'information pertinente, actuelle et utile à la prise de décision pour résoudre, réduire et/ou prévenir ces phénomènes.

Enfin, bien que transformer ces concepts en actions concrètes semble être une tâche ardue, relever ce défi paraît essentiel à l'évolution de la lutte contre le dopage.

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Perspectives for Forensic Intelligence in anti-doping: Thinking outside of the box

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ABSTRACT

Today's approach to anti-doping is mostly centered on the judicial process, despite pursuing a further goal in the detection, reduction, solving and/or prevention of doping. Similarly to decision-making in the area of law enforcement feeding on Forensic Intelligence, anti-doping might significantly benefit from a more extensive gathering of knowledge. *Forensic Intelligence* might bring a broader logical dimension to the interpretation of data on doping activities for a more future-oriented and comprehensive approach instead of the traditional case-based and reactive process. Information coming from a variety of sources related to doping, whether directly or potentially, would feed an organized memory to provide real time intelligence on the size, seriousness and evolution of the phenomenon. Due to the complexity of doping, integrating analytical chemical results and longitudinal monitoring of biomarkers with physiological, epidemiological, sociological or circumstantial information might provide a logical framework enabling fit for purpose decision-making. Therefore, *Anti-Doping Intelligence* might prove efficient at providing a more proactive response to any potential or emerging doping phenomenon or to address existing problems with innovative actions or/and policies. This approach might prove useful to detect, neutralize, disrupt and/or prevent organized doping or the trafficking of doping agents, as well as helping to refine the targeting of athletes or teams. In addition, such an intelligence-led methodology would serve to address doping offenses in the absence of adverse analytical chemical evidence.

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1. Introduction

Forensic science methods can be applied to the specific problem of cheating in sport, whether it is considered criminal, or simply a regulatory breach. Research is currently mostly focused on the analysis of products in biological fluids and the field piles up with analytical tools and methodologies, rather than offering new perspectives and a fundamental look into the phenomenon. Indeed, extensive efforts for the development of more sensitive and selective methods with the latest and most sophisticated technology has been the target of WADA Accredited Laboratories, whereas the community has paid little attention to understanding the phenomenon using strong scientific data and alternative sources of information. A prevalence of blood doping of 14% has been reported by the International Association of Athletic Federations (IAAF) for the entire population of samples collected from elite track and field athletes between 2001 and 2009 [1,2]. Similarly, a prevalence ranging between 10 and 35% has been stated in a survey on doping among German elite athletes in 2008 [3]. However, less than 2% of the ~250,000 blood and urine samples collected yearly are reported to contain a banned substance or to highlight the use of a prohibited method [2,4]. This figure remains quite stable despite a tremendous increase of both doping tests conducted and the sensitivity and accuracy of analytical methods. Thus, only the tip of the iceberg may be perceived by assessing the presence of a prohibited substance or its metabolites or markers in blood or urine specimens of an athlete at a given/specific time. Accordingly, the use, or attempted use, by an athlete of a prohibited substance or a prohibited method is only one of the eight rule violations described in the 2009 World Anti-doping Code [5]. Possession of prohibited substances or methods, trafficking or attempted trafficking of any prohibited substance or method and administration or attempted administration to any athlete in-competition of any prohibited method or prohibited substance constitute rule violations. The investigation of all these

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regulatory breaches have led to little, if any, scientific approaches and are following mostly traditional policing methods despite various reviews on the existence of doping networks or team-organized doping and a black market for prohibited substances [6–9]. At present, the Bay Area Laboratory Co-Operative (BALCO) case which occurred in 2003 in San Francisco and the Operation Puerto which took place in 2006 in Spain remain quite unique in their kind, regardless of the magnitude of doping that they revealed.

Fight against doping needs to consider other scientific avenues to understand and highlight doping offenses in order to exert a preventive or disruptive effect and to support educative or policing decisions by regulatory bodies. The opportunity to gather broader, yet equally relevant, sources of information should be taken in order to bring intelligence to understand doping phenomena and support cases or situations that need action whether in reducing a risk, preventing some substance use or leading to punishment by courts. Anti-doping needs scientific innovation rather than technology innovation and exploring the concept of *Forensic Intelligence* is a most promising, though difficult, avenue. Indeed, performance of complex analytical tools and accreditation has been the center of the debate whereas gathering and exploiting information coming from a multitude of sources has remained obscured by technological debates. Noteworthy, since the 2009 revision of the Code, methods of establishing facts or presumptions of facts related to an anti-doping rule violation may be established by any reliable means. This addition is important as it provides a legal basis for a more flexible fight against doping and for widening the scientific horizon of the field.

Novel *Forensic Intelligence* approaches may further benefit anti-doping and lead to guidelines for the implementation of more specific and efficient models to prevent, reduce or/and solve doping in sport.

2. The fight against doping

2.1. Recent doping cases

A controversial incident occurred in 2003 when three times Track Cycling World Junior champion Mark French was alleged of using prohibited substances use and supply and/or trafficking in the months before the Olympic Games in Athens [10,11]. Traces of glucocorticosteroid were discovered in used drug syringes, needles, empty vials of equine growth hormone and vials of a homeopathic compound found in a rubbish bin in his room at the Australian Institute of Sport cycling training facility. While the Court of Arbitration for Sport (CAS) was unable to prove Mark French's use of growth hormone, he received a two-year ban from competing and a lifetime ban from the Olympic Games. After the first hearing, Mark French appealed the 2004 CAS decision. The panel eventually concluded that there was insufficient evidence to confirm beyond reasonable doubt his use and supply and/or trafficking of growth hormone, and insufficient amounts of glucocorticosteroid in the homeopathic compound to have a significant physiological effect [10,12].

This incident illustrates common issues in the collection and presentation of evidence in court that anti-doping authorities are facing when addressing a wider range of rule violations. Indeed, despite the seizure of prohibited substances and injection material in Mark French's room, the appeal procedure was successful. This case demonstrates the necessity of a different approach for the investigation on doping offenses in the absence of adverse analytical findings (AAFs): forensic science methods may be the best answer.

A more recent example where the handling of injection material followed an appropriate methodology for this type of evidence may be found in the 2007 Rowing World Cup case [7]. During the competition, a plastic bag containing medical equipment was discovered in a waste container. Considering the probability that elite rowers may have used this equipment, the International Rowing Federation (FISA) assigned the Swiss Laboratory for Doping Analyses (LAD) with the investigation. During the initial phase, the substances were identified as non-doping agents. However, the use of an intravenous system is an anti-doping rule violation and syringes, needles and used perfusion material were also found alongside these products [5]. Therefore, the traces of biological material visible inside the infusion tubing were collected for DNA profiling to identify the source donors. With the help of contextual information and the information provided by the physical examination of the packaging of the seized items, the list of athletes to target could be narrowed down to a few individuals, whose DNA profiles were determined after the collection of blood samples serving as reference material. A match between the genetic markers of the traces and the reference DNA profiles provided evidence on the use of a prohibited method by 8 different rowers [7]. Accordingly, the FISA hearing panel sentenced the athletes with a two-year ban from competing but also the coaches and officials of the National Federation from any future involvement with competitive rowing [13].

A more extensive and complex example of the use of police investigation methods was the BALCO incident. Previous to the beginning of the Federal investigation in 2003, an anonymous source provided the University of California Los Angeles (UCLA) Olympic Analytical Laboratory with a syringe containing a designer AAS unknown to anti-doping authorities, which they named tetrahydrogestrinone (THG) [14]. Trevor Graham, a sprint coach whose clients included Marion Jones and Tim Montgomery, was later identified as the anonymous informer. When the laboratory owned by Victor Conte was raided by the U.S. Internal Revenue Service Criminal Investigations Unit and the San Mateo County Narcotics Task Force Records in 2003, large quantities of doping agents of all kinds and materials related to the activity of the laboratory were seized [15]. According to the records, BALCO was responsible for the design of THG in collaboration with a chemist and its subsequent distribution. The investigations revealed that numerous top level athletes were listed in the BALCO files, including Marion Jones (three times Olympic gold medalist and two times silver medalist), Tim Montgomery (100 m world sprint record holder), Barry Bonds (baseball homeruns record-breaking hitter) and Kelli White (100 m and 200 m world sprint champion) [16]. This extensive investigation resulted in the effective prosecution of several athletes for doping offense in the absence of any adverse analytical chemical evidence, which was an unprecedented event in the history of anti-doping [15]. An interesting outcome of this successful collaboration between the United States Anti-doping Agency (USADA), the Internal Revenue Service Criminal Investigations and law enforcement agencies was the recognition of other reliable sources of information for identifying and proving a doping case and its inclusion in the 2004 revision of the Code [17]. Noteworthy, as this scandal raised awareness on the widespread use of doping agents and the limitations of the anti-doping legislation in American professional sports, an improvement of dope testing programs in the U.S. quickly followed and was directly supported by the White House.

Similarly, in 2006, the Operation Puerto shed light on the widespread doping network of Dr. Fuentes, the former Kelme cycling team physician, involving blood doping and transfusion, steroids and growth hormones intake, etc. [18,19] The case arose after the confession of Jesús Manzano on systematic doping during the years he spent cycling under the medical supervision of Dr. Fuentes. As doping is an offense under the Spanish penal law, the Guardia Civil conducted an investigation and seized 200 blood bags

and 105 drugs and doping agents (testosterone, rEPO, growth hormones, insulin, etc.) at the office and home of Dr. Fuentes. Furthermore, a training and doping calendar containing a coded list of approximately 100 names was discovered, which included a majority of top level cyclists and pointed out serious suspicions of blood doping on a number of professional tennis and football players. By overlapping this information with the hematological follow-up of professional cyclists, a few athletes who had previously won the most prestigious races in cycling (Tour de France, Vuelta, Giro, Pro Tour, Olympic Games, etc.) could be identified and convicted of doping. While the collaboration between sport authorities and the Spanish Police in leading the investigation was successful and promising, their joint efforts faced reluctance of the politics to shed light on the rest of the athletes appearing on the list.

All these examples illustrate the use of intelligence to provide investigative leads and to feed the judicial process. However, as these cases would have probably gone unnoticed without confessions or denunciations, they point out the need for a more systematic and thorough use of data to detect and identify both individual and systematic doping in a more proactive fashion. Since anti-doping is mostly isolated from the law enforcement community, the information is fragmented and a platform for the gathering and sharing of knowledge would improve the collaboration between these different authorities. Indeed, information is only useful when exchanged and communicated to relevant people in charge of making decisions and taking actions [20].

2.2. Current approach and limitations

Anti-doping is facing numerous challenges and constraints that prevent the exploration of broader avenues. Actually, today's approach is almost strictly centered on the judicial process, whereas the concepts of detection, reduction, solving and/or prevention of doping stated in the Code also serve health protection purposes [5].

Therefore, anti-doping efficiency should be measured according to the progresses accomplished toward the fulfillment of these objectives and not solely in terms of analytical case numbers. However, the current analysis process lacks originality as it is primarily focused on the detection of prohibited substances and their quantification.

This situation results mostly from an inadequate allocation of human and temporal resources due to recurrent and plentiful scientific, legal and organizational challenges. On a daily basis, WADA Accredited Laboratories are absorbed by the constant and significant flow of blood and urine analyses required by National and Regional Anti-doping Organizations (NADO and RADO), National and International Sports Federations and the International Olympic Committee (IOC) and the regular demands for case support. Additional concerns, related to research and the challenge of developing and validating methods for the long and ever-increasing list of prohibited substances and methods, as well as proficiency testing programs, internal and external quality management audits, ISO 17025 accreditation surveys and the specific requirements of the International Standards for Laboratories (ISL) complete the schedule of Accredited Laboratories [21].

This framework ensures a continuous refinement of state-of-the-art analytical chemistry and biochemistry methods and the ability to detect, identify and quantify every prohibited substance/method appearing in the List. Also, the compliance to a very strict quality management system guarantees a complete follow-up and recording of the chain-of-custody as well as the validity of the analytical results. However, due to these operational aspects, researchers and other partners of the fight against doping may dedicate only little, if any, resources to thinking outside of the traditional judicial process box. As a consequence to this case-by-case and justice-driven approach, anti-doping is missing a strategic vision designed to understand the phenomenon and to develop long-term solutions. Indeed, at present the resources to gain timely and relevant knowledge on doping as a whole and prevent the phenomenon or disrupt organized doping or trafficking of prohibited substances rather than identify isolated cases is limited.

This tunnel vision induces undesirable side-effects on court proceedings themselves. The narrow scope of anti-doping practice by analytical chemistry laboratories comes as a single parameter during court processes. The difference of focus between scientists and lawyers lead to situations that are often perceived as a discussion on the abilities of the scientist rater than the value of the results themselves and an erroneous and partial use of the analytical results. Assessment of an anti-doping rule violation has to remain in the hands of the judge, as the only entity able to weigh every piece of information in the light of each party's plea. However, where scientists should solely be presenting analytical results in a balanced fashion regarding a potential doping offense to bring scientific support to a case, the judge and lawyers wrongly request a personal opinion on the significance of an AAF [22]. This common practice is problematic and results in a questionable alteration of the legal process. Accordingly, scientists become advocates for one side and may unbalance the legal outcome of a trial. Due to the blur around this aspect of the use of science in the legal system, lawyers seek for procedural irregularities or legal flaws and try to challenge the technical expertise of Accredited Laboratories. The whole process shows insufficient emphasis on the physiological meaning of an AAF as the attention of court is somewhat diverted from its original purpose, which may bias the judicial process.

Evidence gathering in anti-doping is usually limited to the collection of a single AAF highlighting the presence of a prohibited substance or its metabolites or markers in a biological sample. Considering the seriousness of the legal outcome, an important step toward a more comprehensive evaluation of cases brought to court could be initiated by collating products of intelligence that originate from broader sources of information. The narrow approach to anti-doping appears questionable when considering the case of both threshold and non-threshold substances. Indeed, part of the List prohibits specific substances above a threshold concentration, which may be misleading depending on the context. If urinary concentrations of xenobiotics are significantly above or below the limit, the evaluation of a potential doping offense is eased, but the closer to the limit, the more complex. In this latter case, considering the analytical uncertainty and potential bias associated with analytical results, detecting and reporting an AAF may turn into a black-and-white process. A similar issue concerns non-threshold substances as an indirect consequence of the Minimum Required Performance Limits (MRPL) rule in force. Indeed, as mentioned in the technical document related to this topic, MRPL have been established as a concentration of a prohibited substance or metabolites or markers that laboratories must be able to routinely detect and identify minimum to ensure that all Accredited Laboratories report results in a uniform way and operate at the same minimal level [23]. MRPL are neither a threshold, nor a limit of detection and AAFs may result from concentrations below the established MRPL values. Actually, WADA recognizes individual capabilities for some laboratories in their ability to identify a wider range of prohibited substances or lower concentrations than other laboratories.

Therefore, the use and strict compliance to threshold concentrations and MRPL may be questionable as this induces a "leap of faith", which demonstrates pernicious effects on anti-doping [24]. Accredited Laboratories may be tempted to develop a habit of not reporting AAFs close to the threshold limit or under the MRPL to

avoid any disputation of their performance in court or simply to be facing difficult cases. Actually, this practice is an involuntary outcome of the lack of intelligence use, which creates a substantial information gap and complications around the interpretation of suspicious findings. A framework of circumstantial information related to the case, the individual physiological variability of the athlete under scrutiny and scientific studies addressing the consumption of the incriminated substance should be considered more systematically during the interpretation of an AAF. Indeed, a tendency may develop to report results within a certain zone of comfort rather than within concentration ranges needing a thorough interpretation of the physiological meaning or prone to questioning on the analytical work's reliability.

As this approach is missing a strategic vision designed to understand the phenomenon, *Forensic Intelligence* might significantly benefit anti-doping.

3. Forensic Intelligence

3.1. General concept

Forensic science is an important aspect of the traditional judicial system, where it serves to lead investigation or to support law oriented questions with scientific information. Analysis can be based on logic besides tools.

Forensic Intelligence embraces this approach and pursues a further goal in the study of criminal activities by bringing a broader logical dimension to the interpretation of trace data. Once collected, the remnants of a litigious activity are thoroughly exploited to detect and provide information on a criminal phenomenon. Subsequently, this gathering of knowledge can support decision-making of *strategic*, *operational* or *tactical* nature in the areas of law enforcement for crime solving, reduction and prevention. *Forensic Intelligence* goes beyond the traditional reactive and case-oriented approach of policing by proposing a more proactive and global response to criminal activities.

Accordingly, Forensic Intelligence endorses different levels of function, namely tactical intelligence, operational intelligence and strategic intelligence (Fig. 1).

These different levels of intelligence operate on an increasing spatial and temporal dimension and overlap to a certain extent rather than being completely distinct processes. In brief, *tactical intelligence* is a reactive approach that aims at providing investigative leads on specific cases to feed the judicial process. At a broader organization level, *operational intelligence* seeks to identify and find solutions to repetitive problems such as serial crimes and criminal organizations. Eventually, *strategic intelligence* is a more comprehensive and proactive approach focusing on criminality at large, trying to point out system vulnerabilities in order not only to reduce and solve criminal problems but also to prevent their development. Logically processing information allows moving from the traditional case-based and reactive approach of policing to more future-oriented and comprehensive decision-making. Therefore, *Forensic Intelligence* proposes a proactive and global response for the prevention and solving of criminal activities rather than focusing on solving a single past event [20].

3.2. Levels of intelligence

3.2.1. Tactical intelligence

The traditional judicial process is composed of three successive phases: the problem to identify and to find, the gathering of evidence to prove the problem, and eventually the trial (Fig. 2) [25].

During this process, *tactical intelligence* supports real-time decision-making of front-line law enforcement officers and brings leads to investigations at a case level. As such, it provides accurate, timely and usable information for crime detection, for identification, localization and arrest of potential offenders, and for gathering of evidence for prosecution in court [27].

Tactical intelligence alone does not seek to bring to light criminal activities that exist either at a scale larger than the individual offender or that constitute repetitive issues [28]. The detection of such criminal problems and their subsequent solution, reduction or prevention require broader sources of information and more thorough exploitation as compared to the short-term case-by-case approach of *tactical intelligence*.

3.2.2. Operational intelligence

Operational intelligence calls for a larger organization level to provide a comprehensive understanding of criminal trends, to ensure a follow up and to help in the coordination of actions (Fig. 2) [28]. This level of intelligence seeks to impact on repetitive problems such as serial crimes, criminal organizations or illicit drugs trafficking by promoting a problem-solving approach endorsing a mid-term oriented and more proactive role than

Fig. 1. Organization and aims of each level of *Forensic intelligence*. From the reactive micro-level of *tactical intelligence* to the crime reduction planning meso-level of *operational intelligence* and the future-oriented and proactive global-level of *strategic intelligence*.

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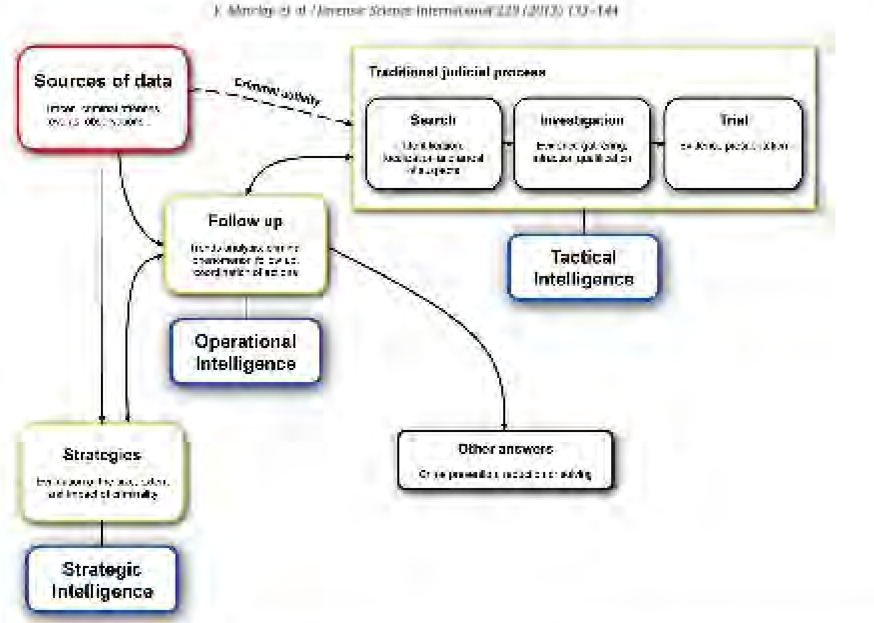

Fig. 2. Structure of *Forensic Intelligence* through logical processing of information supporting *tactical intelligence* to feed the traditional judicial process, *operational intelligence* to plan crime reduction actions and *strategic intelligence* to develop future-oriented and long-term solutions [30].

solely supporting the trial process. Indeed, *operational intelligence* assists decision makers in the deployment and targeting of law enforcement resources as well as in the planning of actions for crime reduction or prevention [29].

With the help of exploratory, statistical and visualization methods related to crime analysis, extensive amounts of information saved in a structured memory are logically processed to detect geographical and/or temporal problems, to determine the type of offenders and to identify criminal patterns, or so-called modus operandi. As this analysis process is iterative due to the constant flow of new information filling the memory, *operational intelligence* supports intelligence-led policing with constantly refined and updated knowledge.

3.2.3. Strategic intelligence

Strategic intelligence operates at a third and more global level of organization. Criminality is a complex phenomenon constantly evolving over space and time due to changes in demographics, economics, politics, environment, etc. Therefore, *strategic intelligence* encompasses these sources of information in a criminology perspective and sub-levels of *Forensic Intelligence*. This reasoning process is a multivariate approach that asks for the description and understanding of criminality as part of an environment in perpetual change (Fig. 2) [28]. *Strategic intelligence* is future-oriented and proactive as it intends to foresee the development of criminal threats in order to plan crime reduction of emerging issues or, within realm of possibility, to plan crime prevention by identifying and resolving system vulnerabilities that allow the apparition of criminal problems. Indeed, an interesting outcome of *strategic intelligence* is the ability to identify areas where policing and harm minimization actions may achieve positive results [20].

This approach seeks to impact on the phenomenon as a whole rather than on specific criminal activities. Therefore, *strategic intelligence* can contribute to decision-making from the law enforcement level up to the political level to design long-term problem-solving policies as well as preventive and educative actions or programs.

Noteworthy, *operational* and *strategic intelligence* may overlap to a certain degree, depending on the size and seriousness of the phenomenon under scrutiny. As both share a vast array of sources of knowledge, these different forms of intelligence are interactive rather than distinctive processes.

3.3. Structured memory

Forensic Intelligence is embracing a number of scientific disciplines to detect, collect, describe and compare traces related to litigious activities, which once collated with other sources of information and exploited using crime analysis tools provide a better understanding of criminal patterns. Considering criminality as a global phenomenon rather than isolated cases, *Forensic Intelligence* does not limit to the study of physical, biological or chemical evidences to answer source and activity questions and to support decision-making at *tactical, operational* and *strategic* levels.

Actually, the operation of Criminal Intelligence depends on a structured memory of traces and information to gather knowledge on a criminal phenomenon. As an organized repertoire of systematically and continuously updated or possibly applied inferences reasoning, the memory represents the knowledge we have at a certain time about the criminality under consideration: current criminal problems or patterns, serial crimes, cases linked, etc. (Fig. 3).

Information on a criminal phenomenon is first acquired and then integrated into a follow up framework. Characteristics of these new data are extracted and sorted out prior to being merged with previously memorized information, allowing drawing

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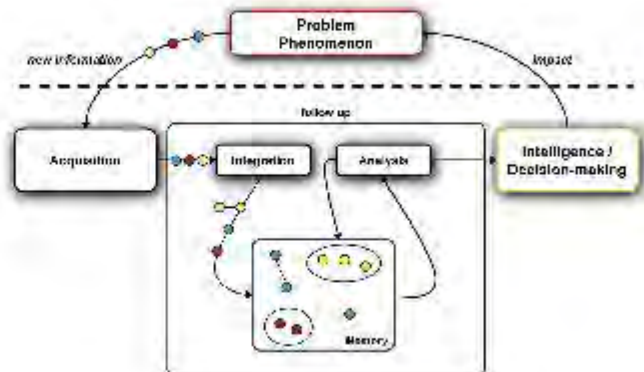

Fig. 3. Logical processing of new information acquired and integrated to a structured memory containing previously stored information [30]. The analysis is an iterative and cyclic process that allows transforming these raw data into timely and usable information to support decision-making in order to impact on the criminal phenomenon under scrutiny.

inference structures that reveal a network of hypothesized links. This reasoning process is entirely based on postulating and testing assumptions on the relation between items organized in the structured memory.

While finding links may appear somewhat straight-forward, linkage blindness may occur due to the increasing complexity of the memory, the incompleteness of the data and constraints of political, legal or organizational nature [31]. Indeed, as the structure of the memory becomes more sophisticated with its expansion, the network of links may become a blur and links between objects, individuals and/or events may be missed. Therefore, as an initial phase of the analysis, the memory as a whole or as sets of smaller size may be visualized using relational diagrams or maps to reduce the splitting of information (Fig. 4).

The analysis of the memory is an interpretative step where each new piece of information may confirm the predicted truth-value of hypothesized links and/or result in the connection of sets of information originally considered as distinct. Conversely, the outcome of this logical process may question or even exclude the existence of links previously assumed. Depending on the perspective, new pieces of information may serve for immediate use or may be stored to feed future inference processes. These new

Fig. 4. Simplified example of a relational diagram to visualize the links between objects, individuals or/and events involved in criminal activities such as drugs or/and stolen vehicles trafficking.

Fig. 5. The logical analysis cycle of the information stored in the structured memory [31].

items may be organized in a short-term memory for direct exploitation on a specific criminal case, or may be integrated to a long-term memory to depict criminal trends or series or to identify system vulnerabilities.

Therefore, the follow up process is iterative and dynamic as organizing and scrutinizing the memory in order to infer new information is most likely going to modify the picture of a phenomenon and to result in an update of the memory (Fig. 5).

The coherence of the memory is ensured by this logical methodology. An interesting outcome is the ability to synthesize a set of information describing the phenomenon under scrutiny. This reasoning process provides decision makers with a real-time insight into criminality and accurate and usable information to support investigative leads, to help in the coordination of actions and in the designing of policing operations. Such measures may impact on different aspects of criminality by reducing, solving and/or preventing the phenomenon. As a result of these law enforcement actions and policies, but also due to environmental variations, isolated authors up to criminal organizations may adapt their modus operandi in order to pursue their activities or else may disappear. This shift in criminality requires the acquisition of new information to feed and refresh the Criminal Intelligence process in a cyclic mode in order to ensure up-to-date knowledge.

Noteworthy, the concept of intelligence is a particularly versatile approach as illustrated by its application in the economical, sociological or demographical fields.

4. Perspectives for Forensic Intelligence in anti-doping

The concept of *Forensic Intelligence* previously described may be transposed into *Anti-Doping Intelligence* by relying on a similar methodology and shifting the paradigm to the problem of doping in sport (Fig. 6).

At *tactical* level, where *Forensic Intelligence* is focusing on the individuals, *Anti-Doping Intelligence* would be focusing on the athletes. Likewise, at *operational* level, the attention on serial crimes and criminal organizations would be switched to organized doping and the trafficking of doping agents. Eventually, at *strategic* level, the emphasis would be put on understanding the doping phenomenon instead of criminality.

Considering the considerable gap between the estimated prevalence of doping reported in different surveys and the annual WADA statistics on AAFs, there is room for a more thorough exploitation of information to make the fight more efficient [1–4]. Therefore, a series of perspectives for *Anti-Doping Intelligence* will be discussed in the following paragraphs and examples will be presented to illustrate this concept.

4.1. Detection and monitoring of doping phenomenon

As mentioned earlier, the primary focus of anti-doping is the detection and quantification of exogenous substances found in biological fluids and on source inference questions for the

Fig. 6. Transposition of the *Forensic Intelligence* concept into *Anti-Doping Intelligence*.

distinction between the endogenous or exogenous origin of naturally occurring substances. However, this aspect is only one of the very end-products of the whole doping phenomenon.

Indeed, products of *Anti-Doping Intelligence* may help in the detection and description of a potential, emerging or existing, yet unnoticed, trend in the consumption of doping agents. The information may come from a variety of sources, including sociological studies on substance use or abuse, a notice of release from the pharmaceutical industry on a new medication with performance-enhancing properties, statistics of law enforcement authorities on substance trafficking or the reporting by the medical staff of a sport's team of a growing trend in the consumption of a particular substance, etc. This knowledge would also be relevant as prevalence of doping and substances use vary between sports, national and international level, genders and also across space and time [3].

Thereof, sociological studies and surveys on the consumption of doping agents and other drugs would be very informative tools to inform on the size, seriousness and evolution of a problem. Through logical processing of these elements, the threat on all different aspects and values of a doping-free sport may be assessed and the mechanisms behind doping better understood. The measurement of this statistic is a recurring topic of under-estimated value and prone to legal limitations. Indeed, as mentioned in the ISL, samples must be analyzed to detect prohibited substances appearing on the List and no sample may be used for any other purpose without the athlete's written consent [21]. A complete removal of all identification means must be ensured before using samples for research purposes, in order to prevent any traceability back to a particular athlete. As obtaining the consent of the athletes in the first place is extremely challenging, this legal mention makes it very difficult for exploratory epidemiological studies in anti-doping. Therefore, this rule greatly complicates the discovery of trends in the consumption of unlisted or designer drugs with performance-enhancing properties, especially as an exemption is always difficult to justify.

Nevertheless, this knowledge would be helpful for implementing strategies adapted to the specificities of each situation. Indeed, finding a unique solution to all problems appears somewhat utopist, where designing multiple and tailored policies appears more fit-for-purpose. This might result in the inclusion of a substance or method to the List or the Monitoring Program, the adjustment of existing policies, the development of a preventive or educational program, or, conversely, the decision not to legislate or to cancel the current regulation as the threat may be considered as insignificant. As a consequence, such a dynamic and intelligence-led approach may prove efficient at providing a more proactive response to any potential or emerging doping phenomenon or to address existing problems with innovative actions or/and policies.

In turn, longitudinal prevalence studies would allow assessing the efficiency of anti-doping programs by monitoring the evolution of a phenomenon.

4.2. Longitudinal and indirect approaches

As illustrated with the 2011 WADA/UCI v. Alberto Contador Velasco & RFEC case, the source of low-levels of doping agents, in this case clenbuterol, is difficult to ascertain due to the probability of environmental or food contamination or any other potentially explanatory reason [32,33]. Since similar situations tend to happen more frequently, especially due to the constant increase in sensitivity of analytical instruments, a global approach in anti-doping might become essential. This situation illustrates the crucial need for collaborative research between experts in the fields of analytical chemistry, endocrinology, genetics, pharmacology, physiology and sports medicine to understand the complexity of the doping phenomenon within a forensic science framework [17]. A structured memory integrating analytical results and longitudinal monitoring of biomarkers with physiological, epidemiological, sociological, circumstantial, etc., information, as well as products of intelligence, may provide a logical framework enabling fit-for-purpose decision-making in anti-doping.

Considering *tactical intelligence* perspectives, the structured memory attached to every athlete would integrate atypical findings or AAFs and all doping-related information to help refining the targeting of athletes, which would increase anti-doping efficiency. In addition, the memory would also serve to strengthen the use of other parameters than pure analytical chemistry results in support of cases brought to court. Actually, when considered with other pieces of evidence or products of intelligence, a xenobiotic detected at a concentration below the threshold or the MRPL could be relevant in both detecting and assessing a potential rule violation. Additional information saved in the memory may include the results of previous doping tests, individual physiological particularities of the athlete, *Anti-Doping Intelligence* information, etc. As an example, the presence of one or several exogenous prohibited substances below the threshold limit may have been repeatedly highlighted for an athlete. In this context, each individual test does not consist in a doping offense. However, considering a longitudinal perspective, this is an interesting indicator of a potentially continuous substance intake and doping behavior. Concerning endogenous compounds such as anabolic androgenic steroids (AAS), human growth hormone (HGH), human chorionic gonadotropin (hCG) or recombinant erythropoiesis (rEPO), concentrations in biological fluids may vary naturally quite significantly, regardless of a substance intake. As an illustration, the influence of genetic factors, ethnicity, sex, diseases or diet on concentration levels and/or carbon isotopic ratio ($^{13}C/^{12}C$) of AAS molecules has been reported in the literature [34,35]. In this context, a simple concentration measurement may not be sufficient to distinguish between the endogenous or exogenous origin of the substance and assessing other biomarkers may be necessary. On the one hand an athlete may dope with an endogenous substance and yet go unnoticed due to a naturally decreased physiological response to a substance or a naturally elevated rate of metabolism resulting in a very short detection window. On the other hand, an athlete may show a naturally elevated physiological variability in the concentrations of an endogenous compound and meet the criteria of an AAF without any substance intake. The situation of the endogenous compounds underlines the need to set individual threshold limits for athletes or/and to develop indirect approaches where biomarkers or/and longitudinal monitoring would be more informative to determine the origin of a substance and to increase the detection window.

Accordingly, the Athlete Biological Passport aims at the detection of blood doping in the form of blood transfusion or the use of erythropoiesis-stimulating agents such as rEPO [36]. The hematological profile has brought pioneer work as a first step in the development of a logical and inferential approach for the interpretation of indirect markers of doping. Indeed, the longitudinal monitoring of multiple blood parameters has initiated an important move toward the elaboration of models to interpret data based on transparent and sound logical processes. This approach provides a more comprehensive evaluation of observing changes in levels and ratios of blood parameters if an athlete is doped rather than clean, by taking into account natural variations over time to set personalized and adaptive confidence intervals and limits for each athlete [37]. As such, the likelihood of observing significant changes in an Athlete Biological Passport due to blood doping is balanced with several potential natural causes known to modify

the erythropoiesis and other blood parameters. The athlete's hematological profile allows refining the targeting of athletes by generating alerts when abnormal variations are observed. This is most helpful to increase the efficiency of doping controls and to deter blood doping, or to take disciplinary actions when an Adverse Passport Finding (APF) is declared [38].

Likewise, steroidomics, a specific area of metabolomics, aims at the discovery of biomarkers of direct doping with synthetic analogs of AAS or indirect doping with selective estrogen receptor modulators (SERMs), aromatase inhibitors or hCG. The steroid profile is constituted of concentrations and ratios of endogenous steroidal hormones, as well as related precursors and metabolites. The modification of this profile, due to either direct or indirect stimulation, is processed through chemometric models taking into account the global steroid metabolism to distinguish between a natural physiological condition and steroids misuse [39]. Interestingly, the strategy behind the identification of indirect markers allows highlighting physiological variations due to doping independently from the type of prohibited substance or method used. Considering the constant flow of new therapeutic agents and designer steroids, yet unknown to anti-doping authorities, pouring on the marketplace, this aspect is particularly advantageous for overcoming the limitations of current analytical methods. In parallel with the Athlete Biological Passport, the current development of a urinary steroidal passport integrating the longitudinal monitoring of indirect markers of steroids misuse shows promising preliminary results as a way to extend the detection window and increase the sensitivity of the tests [39–41]. As a consequence, the rate of false-negative results may be greatly reduced. Alternatively, the risk of false-adverse analytical finding resulting from a single spot blood or urine test may be significantly reduced as such indirect approaches can highlight a natural cause to physiological variations exceeding the general population limits. Bearing in mind that the burden of proof is on the testing authority, keeping this risk at a strict minimum is crucial [36].

Interestingly, the hematological and steroidal passports may also serve a further purpose in the evaluation of the performance enhancement after substance misuse in order to assess the degree of gravity of a doping offense, besides a general overview of the health of the athlete himself. Indeed, an athlete doping with low-levels of a prohibited substance might not show a significant physiological response, hence benefit from a limited doping effect, as opposed to another athlete involved in heavier misuse. With regards to these scientific considerations, the reasoning around doping may evolve toward more equitable and flexible justice mechanisms. Actually, compared to criminal law, the anti-doping legislation defined by the Code is somewhat rigid as neither does it contain a scale of penalties based on the gravity and circumstances of the offense nor does it leave room for such distinctions.

Therefore, *tactical intelligence* would help to support specific cases on strong scientific grounds and *operational intelligence* would allow improving the detection of doping with a refined targeting of athletes.

4.3. Organized doping and trafficking of doping agents

With more than 200 substances in the 2013 WADA Prohibited List, trafficking of doping agents is an attractive and lucrative business of probably underestimated dimension that may follow complex pathways and involve criminal organizations [8,42,43].

Supply sources may vary quite notably depending on the type and legal status of a substance. Indeed, pharmaceutical preparations containing doping agents such as pseudoephedrine may be readily available over the counter and require no prescription. In contrast, pharmaceutical products for therapeutic use, like rEPO or HGH, may require a prescription or may also be counterfeited by clandestine laboratories or diverted from production stocks of the pharmaceutical industry and supplied on the black-market [15]. Therefore, both original and counterfeit doping agents are being trafficked. In any case, a number of illicit products sold by drug dealers, such as cocaine or amphetamines, are also doping agents.

Therefore, doping is a complex phenomenon considering the vast variety of substances, supplied through both legal and illegal trading routes, and the extensive connections between the people involved in the network. While doping may appear as the doing of an athlete on its own, it always involves one or several entities in the supply of doping agents or in their use, whether the friends and relatives of the athlete, or the medical staff, manager and teammates of a sport's team, or chemists, biologists and pharmacists, or pharmaceutical industries and clandestine laboratories, or criminal organizations, drug smugglers and dealers (Fig. 7).

Bearing in mind that in most parts of the world there is no regulation on the production and trafficking of doping agents,

Fig. 7. Doping network and the diversity of potential links between its different entities.

there are very fertile grounds for the development of clandestine pharmaceutical plants. On the whole, industries in Thailand, China, India and Russia play a leading role with numerous production sites and well-established trading routes [8]. Actually, statistics on seizures of doping substances indicate China and India as the fastest growing source of suppliers of the international gray-market, as reflected by their ever-expanding pharmaceutical industries. In short, production in Thailand, China, India, Russia, Greece and Mexico account for approximately 55% of the global distribution of doping agents. Besides, a hint on the involvement of the industry in this business may be found in the overproduction of rEPO, which is estimated at about five to six times the real worldwide treatment requirements. Indeed, a panel of experts supported by governmental institutions pointed out a significant imbalance between the production of pharmaceuticals with doping properties, namely rEPO, HGH and testosterone, and the therapeutic requirements of patients [8].

According to the statistics on seizures of doping agents, the global production of AAS might be estimated to reach approximately 700 tons a year, which is enough to supply 15 million people annually. In particular, approximately 70 tons of testosterone are produced to meet the requirements of 1.5 million people each year. Similarly, rEPO and HGH production reach approximately 34 million phials a year, enough to supply up to 2 million people [8]. The diffusion of doping, which accounts for approximately 15.5 million people, does not concern only top-level athletes, but also various categories of individuals. As mentioned in the Donati report on World Traffic in Doping Substances, doping as a whole is constituted of approximately 35–37% athletes of all levels, 38–40% body-builders and gym-goers, which include private surveillance agents, 4–6% military and police forces, 1–2% people involved in show business and 15–20% false treatments [8].

The Internet has brought new horizons to the trafficking of doping substances in simplifying and securing its global development. As mentioned earlier, the lack of regulation on the production of doping agents in most countries of the world and the multiplication of online "pharmacies" ensure the ease and safety of this gray-market, which translates into a constant growth. Indeed, there is significantly less risks for the producers and traffickers of doping substances to leave the stocks in the production country and send them by postal service in small quantities than to load a truck with large quantities and undertake a dangerous journey in the open and through customs to reach the destination country.

Several different forms of trafficking have been identified [8]. The traditional movement of doping agents consists in loading large quantities onto a conveyance for transportation to intermediate locations, where a part of the shipment is delivered, while the rest continues to the next destinations. This form used to be the most popular previous to the e-commerce era and is becoming less frequent nowadays. A more common form, which is similar to the traditional one, relies on the postal service system for final dispatch after transportation to the intermediate location. The advantage is to allow the gathering of different doping substances in a specific place to build up online "pharmacies" which will send small packages to the purchasers. Eventually, the most straightforward and fastest growing form involves stocking doping agents directly on-site or in the production country prior to their shipment to the online purchasers.

Although the supply of doping substances may seem to be set on the individual scale of the athlete, its major dangers and risks lie in organized doping and in the market largely linked to and dominated by international criminal organizations. The Italo-American Mafia has been in control of the trafficking of AAS and HGH in the United States up until the mid 90s and the onset of the so-called Russian Organized Crime (ROC) as a predominant actor on the gray-market. Soon afterwards, Asia's share into the production and worldwide trafficking of doping substances considerably expanded to replace the Russian Mafia as the leading power of the gray-market. Noteworthy, in several instances the trafficking of illicit drugs and doping agents as well as counterfeit medicines has been found to originate from the same criminal organizations [44].

Despite the awareness of law-enforcement agencies on the doping problem since several decades, linking the different entities of a doping network remains a difficult and unexploited field, especially because only a very few countries consider doping as a criminal offense under the penal law. Indeed, doping in sport is a matter of public criminal law in Spain, Italy and Belgium, defining a legal framework for criminal justice mechanisms and the use of police investigations [11]. Alternatively, countries such as the United States and Australia define doping in sport as a matter of private law. Therefore, inquiries into rule violations are carried out between the International Sport Federation and the athlete, prior to being transmitted to the National Anti-Doping Organization serving as an administrative body, or to a private arbitration panel, most likely the CAS. In contrast, the situation in France since the revision of its anti-doping Act in 2006 is a compromise between a reinforced administrative process and the criminalization of specific activities supporting the doping of athletes [45]. As a result, the angle at which the phenomenon of doping is addressed in each country of the world will reflect those different legal systems. On the one hand administrative processes generally aim at prosecuting athletes violating an anti-doping rule, where the use of criminal justice mechanisms allows additional investigations on organized doping and taking action against the people involved other than the athletes.

Except in the specific jurisdictions mentioned above, criminal organizations live through and enjoy the relative safety created by the lack of collaboration, and legal ground in the first place, which persists in sharing relevant information held at different levels by the WADA, National and Regional Anti-Doping Organizations (NADO and RADO), National and International Sports Federations, the IOC, WADA Accredited Laboratories, national customs and border agencies, national police services, the European Police Office (EUROPOL) and the International Criminal Police Organization (INTERPOL). Collected and structured information integrated in a framework developed in Criminal intelligence analysis may prove useful and potentially and particularly efficient for identifying systematic doping or trafficking networks and to neutralize, disrupt or prevent these activities [27]. While relevant information is in possession of anti-doping authorities and their partners, the general lack of legal regulation is a major obstacle for implementing *Anti-Doping Intelligence* analysis. Indeed, the exchange of sensitive information requires a legal framework, which may be found in the inclusion of possession and/or trafficking of prohibited substances to national criminal codes. With few exceptions, partners of the fight against doping are non-governmental bodies, which limits their freedom of action in addressing the phenomenon from a more global perspective. On a side note, when it comes to allowing financial and human resources, governments are facing a question of priority compared to more crucial issues, such as healthcare, education, employment or criminality but also in very common instances water and food supply, poverty or human rights. One cannot expect countries with such persistent, and sometimes urgent, problems to dedicate all the necessary resources and to support equally what appears to be a minor concern. Although it may be argued that an efficient anti-doping policy may provide and safeguard good health for the most active youth of a country.

A *Forensic intelligence* approach has been developed at the School of Criminal Justice (ESC – Ecole des Sciences Criminelles) in Lausanne, Switzerland, to help understand and fight illicit drugs trafficking as well as in the detection and description of criminal organizations involved in the counterfeiting of pharmaceuticals. The illicit product itself is the single direct physical object resulting from a trafficking network and constitutes, therefore, the only direct and major element linked to the criminal organization. It carries information that remains an indicator of source and activity and is decisive in the perspective of decoding the structure of the phenomenon. Drug profiling is this process of extracting the physical and chemical profiles for use in the application of policies against illicit drugs trafficking [20]. Chemical characterization of prohibited substances, physical examination of the packaging and other circumstantial data (time, location, etc.) are stored in a structured memory for further exploitation. Then, inference models are built upon these data to link drug seizures, highlight distribution networks and identify sources of supply in order to support decision-making and prioritizing [46]. Considering an *operational intelligence* perspective, similar chemical or/and physical profiles may bring to light a link between separate drug seizures or trafficking cases [20]. From a *strategic intelligence* perspective, this information may serve to visualize routes or networks of trafficking or identify tendencies in the use and trafficking of illicit drugs.

Transposing this approach to anti-doping, schemes indicating relations between individuals and/or substances would greatly improve the understanding of the structure of doping activities and the success rate in tackling the traffic of prohibited substances and organized doping. Relational diagrams are a common visualization method to help in the process of describing the links that constitute the backbone of the phenomenon. Collection and sharing of sensitive information are essential to have a real time evaluation of the size, evolution or mechanism of doping and to produce intelligence.

The strategy behind *Anti-Doping Intelligence* may help refine the targeting of athletes or teams, the identification of doping promoters and the deployment of adequate operations to dismantle core ramifications of doping networks. The overall advantage of this approach is to elicit information of a different but complementary nature than currently that may prove efficient in taking *strategic*, *operational* or *tactical* measures whether these are preventive or instrumental of criminal proceedings. Also, this scientific methodology is more global and comprehensive, rather than strictly focused on laboratory analyses and the traditional judicial process.

As mentioned previously, athletes represent approximately a third of the population using doping agents. Considering that professional athletes bound to the Code's regulation are a minority and in most instances the only group of people under continuous medical supervision, it is obvious that the general population of users is at high risks. Indeed, they tend to show little knowledge or care about the prescribed daily dose and underestimate the side-effects of regular use. In addition, as these people do not fall under the legislation of anti-doping, the trafficking organized around this huge part of the market is considerably facilitated. Therefore, the politics should consider doping as a public health issue and not simply as a problem within the professional sports community. Raising awareness on the reality of the situation would help to legitimate the establishment of legal grounds necessary to the implementation of Anti-Doping Intelligence.

5. Conclusion

Anti-doping needs to embrace an explorative attitude and diversify its scope of research toward *Forensic Intelligence* to progress in the completion of its numerous missions, whether in the solving, reduction and/or prevention of doping. Therefore, thinking outside of the judicial process box is absolutely essential for a more comprehensive and efficient approach of the fight against doping. *Forensic Intelligence* would not only provide accurate and timely information to support case-by-case evaluation during the traditional judicial process, but also to feed decision-making to address the doping phenomenon on a scale which may become global.

While doping tests are considered as the primary and almost exclusive source of information, more diversified avenues have to be considered and integrated into a structured memory of the doping problem. This follow-up framework would contain information on all different sub-levels of the phenomenon, including the anti-doping history of each athlete's, to help the visualization and understanding of the problem. The product of intelligence resulting from the logical processing of this structured memory should offer real-time knowledge on any doping problem to support intelligence-led control of *tactical*, *operational* or *strategic* nature in a proactive rather than reactive fashion.

In conclusion, *Forensic Intelligence* offers a promising reasoning framework to put in light all the anti-doping rules described in the Code instead of merely limiting the fight to a single rule, which is the use or attempted use of any prohibited substance or method. Such an intelligence-led approach would benefit anti-doping as a whole, including the fight against organized doping and the trafficking of doping agents.

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Use of forensic investigations in anti-doping ☆

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ABSTRACT

The fight against doping is mainly focused on direct detection, using analytical methods for the detection of doping agents in biological samples. However, the World Anti-Doping Code also defines doping as possession, administration or attempted administration of prohibited substances or methods, trafficking or attempted trafficking in any prohibited substance or methods. As these issues correspond to criminal investigation, a forensic approach can help assessing potential violation of these rules.

In the context of a rowing competition, genetic analyses were conducted on biological samples collected in infusion apparatus, bags and tubing in order to obtain DNA profiles. As no database of athletes' DNA profiles was available, the use of information from the location detection as well as contextual information were key to determine a population of suspected athletes and to obtain reference DNA profiles for comparison.

Analysis of samples from infusion systems provided 8 different DNA profiles. The comparison between these profiles and 8 reference profiles from suspected athletes could not be distinguished.

This case-study is one of the first where a forensic approach was applied for anti-doping purposes. Based on this investigation, the International Rowing Federation authorities decided to ban not only the incriminated athletes, but also the coaches and officials for 2 years.

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1. Introduction

The World Anti-Doping Code establishes a harmonisation of the anti-doping rules across all sports and countries in the world [1]. It provides a framework for rules, regulations and practice of sport.

Doping is defined in this document through a number of articles, in Sections 2.1–2.8. Despite the broad scope of this code, the fight against doping is mainly focused on the first definition, which is the detection of a prohibited substance in an athlete's biological sample. For this purpose, blood and urine specimens are collected in and out of competition and submitted to a variety of analytical tests designed to highlight the presence of a banned substance (e.g. stimulants, anabolic steroids, exogenous erythropoietin (EPO), corticosteroids, and monitoring of blood transfusion) [2].

Noteworthy, other doping offences include the use or attempted use by an athlete of a prohibited substance or method, possession of prohibited substances or methods, trafficking or attempted trafficking in any prohibited substance or methods, or administration or attempted administration of any prohibited method or substance. However, these violations of the Anti-Doping Code are barely investigated as traditional analytical methods cannot provide relevant information on these offences.

Since such issues correspond closely to criminal cases under forensic investigation, relying on a similar approach may benefit the fight against doping to help assessing potential violation of these rules. Indeed, collecting items, examining evidences and interpreting results other than dope testing biological samples would allow assessing the use, administration and possession of prohibited substances or methods by drawing links between seized prohibited substances and/or medical equipments and an athlete or his entourage [1]. Also, a drug intelligence approach used for tackling drug-trafficking networks could also be applied to identify and dismantle doping products-trafficking networks to which an athlete or his entourage may be linked [3].

Nevertheless, while these techniques have a great potential in the fight against doping, their use remains marginal. Therefore, this paper describes a recent doping case where a forensic approach proved successful in providing evidence that led to suspension for

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anti-doping violation rules of several athletes and related technical staff. As such, investigation methods novel to the field of anti-doping will be presented in this case study.

2. Case report

In July 2007, during the Rowing World Cup on the Rotsee Lake (Lucerne, Switzerland), a plastic bag containing different types of medical equipment was found by a local resident in a waste container. As a witness had seen a team official throwing away the incriminated plastic bag into the compost bin, he decided to report this to the International Rowing Federation (FISA). Considering this fact and as the probability that elite rowers were involved in the use of these equipments was high, the Federation decided to transmit the material to the Swiss Laboratory for Doping Analyses (LAD) for investigations (Fig. 1). FISA asked the LAD to undertake extensive analyses to investigate on a potential anti-doping rule violation and to evaluate involvement of suspected athletes in this case.

The first aim was to establish if there was a violation of anti-doping rules. The drug present in the plastic bag consisted of 12 bottles of Neoton®, 10 bottles of Esafosfina®, a bottle of aminocaproic acid, 4 vials of Panagin®, 2 vials of inosine and 2 boxes of Biotad® tablets. Analysis on the composition of these products revealed that there were compounds used for faster recovery only, and none of them could be considered as doping agents. However, 4 syringes, 4 needles and 13 used intravenous infusion items were found alongside these products. According to the World Anti-Doping Code, the use of an intravenous system constitutes a violation of the anti-doping rules [2].

As red residue was visible inside the infusion tubing, potentially corresponding to blood traces, it was decided to conduct genetic analyses on these biological samples after collection, in order to obtain DNA profiles that could later be compared to DNA profiles from suspected athletes (Fig. 2). However, since no database of athletes' DNA profiles was available, and to avoid profiling all rowers who took part in this competition, an evaluation of the contextual information available was crucial. Indeed, this kind of information was taken into account when determining which athletes should be targeted. As a first indication, the medical material was found in a rubbish bin located behind the hotel where two federations' teams were staying, namely Nation A and B. Moreover, the drug packaging and the plastic bag containing it provided additional and relevant information. Indeed, the inscriptions were written in Cyrillic alphabet and only Nation A was from a country using this alphabet (Fig. 3). According to these observations, the FISA decided to target only athletes from this nation.

Thereafter, the LAD submitted all parts of the perfusion systems (bottles, syringes, plastic tubes and needles) containing biological samples to the Forensic Genetics Unit (UGF). This material was analysed for DNA profiling in order to identify the source donors of the blood collected on the perfusion systems. Afterwards, FISA decided to collect anti-doping tests blood samples on rowers of the Nation A team to obtain reference DNA profiles for comparison.

Fig. 2. Red residues inside the tubing systems.

3. Materials and methods

DNA extraction was performed on 10–100 μL of liquid blood collected on the infusion tubing. If dry, the blood was directly rinsed with the buffer used for DNA extraction. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) following the manufacturer's instructions. DNA extracts were concentrated to about 25 μL using Microcon 30 spin columns (Millipore AG, Zug, Switzerland). They were quantified with a real-time PCR in order to set-up the DNA amplification protocols. This was performed with the Quantifiler Human DNA Quantification kits using a qPCR ABI 7300 according to the manufacturer's instructions (Applied Biosystems, Zug, Switzerland) in half reaction volume. The mean DNA concentration of the blood stains was 17.59 ng/μL, ranging from 0.04 (dried blood in a plastic tube) to 66.84 ng/μL (liquid blood in a needle). Reference samples were analysed with the same protocol but in a dedicated room.

DNA amplifications were carried out with the AmpFℓSTR® SGM Plus® PCR Amplification Kit from Applied Biosystems, following the manufacturer's instructions, in half reaction volume. This kit amplifies 10 Short Tandem Repeat loci (D3S1358, VWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01

Fig. 1. Overview of the material present in the dustbin.

Fig. 3. Cyrillic alphabet present on several drugs packaging.

and FGA) plus the gender marker Amelogenin. Amplified DNA was analysed with an ABI 3100 Genetic Analyzer from Applied Biosystems following standard procedures. For each stain and reference sample, DNA profiles were validated with a second result obtained either with an independent DNA extraction or with two independent amplifications of the same DNA extract. The least concentrated sample was amplified with an increased number of PCR cycles (34) to enhance the sensitivity of the amplification. For this sample, a consensus DNA profile was built from 5 amplifications using the guidelines from Castella et al. [4].

4. Results and discussion

4.1. DNA analyses

Forensic methods can provide useful tools in investigating the possible violation of anti-doping rules by using techniques based not only on the analysis of a sample provided by the athlete, but by the presence of other evidence. Only a few cells are necessary to establish a DNA [5,6]. Indeed, sufficient biological material might be collected on the neck of a drug flask, inside an infusion tube, syringe, blood bag or urine sample to provide a DNA profile. As a matter of fact, the two main matrices for doping analyses, which are urine and blood, are compatible with DNA profiling. Therefore, after comparison with a reference sample of an athlete, the source of a biological sample may be assessed.

Presently, forensic genetics has already been used in anti-doping to identify individuals by their DNA profiles obtained from urinary extracts. In most of the cases, the aim of DNA profiling was to demonstrate that a urine sample really belonged to an athlete or conversely to investigate whether an athlete really gave genuine urine and not a negative urine hidden in a pocket [7,8].

Although DNA profiling is a very powerful tool for identification purposes, its potential for the fight against doping remains widely unexploited. Indeed, it may provide relevant evidence on violation of anti-doping rules where traditional techniques would prove totally inefficient.

4.2. Application to the case

In the particular context of the case, the motivation for FISA to use DNA analysis was to determine if one or more anti-doping rule violations occurred. According to the examination of the seized material and the preliminary analytical results, further investigations had to focus on the possible use of a prohibited method rather than a prohibited substance. Also, FISA had to evaluate whether the use of the intravenous infusion equipment was medically justified or not. Indeed, infusion is only allowed for legitimate medical treatment but prohibited for enhancing recovery [2]. However, as the seized substances were not determined as doping agents, direct detection and quantification methods in doping samples would not prove relevant. In consequence, obtaining the identity of the persons who used these equipments for doping purpose was mandatory.

As a first step, the location of the medical equipment allowed focusing on a limited number of athletes. Accordingly, examination of the Cyrillic alphabet appearing on several items, including two bags containing drugs, syringes, ampoules, perfusion bottles, packing tape and infusion systems, provided useful information for reducing the population of athletes potentially incriminated. Indeed, this alphabet is found in Eastern European countries such as Bulgaria, Russia or Ukraine. As Nation A was using the Cyrillic alphabet, it was decided to conduct tests on athletes of their team. These two pieces of information were very important for limiting the number of analyses required and to shorten the time necessary to find the perpetrators. If the perfusion systems and the drug packaging had been transmitted to the LAD alone, reducing the number of suspects to such a low number would have been difficult and investigations would need to have been extended to many more athletes (Fig. 4).

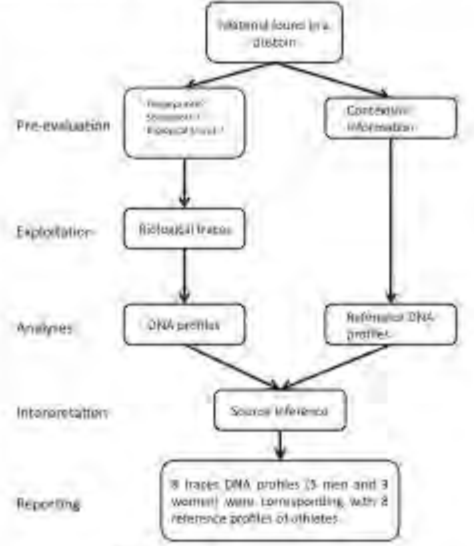

Fig. 4. Forensic investigation approach.

Subsequently, DNA profiling was conducted by the UGF on 10 biological samples collected on the parts of perfusion systems (bottles, syringes, plastic tubes and needles) and 30 reference blood samples from athletes of Nation A present at the competition. It was possible to determine 8 different DNA profiles coming from the different samples and due to the presence of a gender marker, 5 of these profiles were determined as male and 3 as female (Fig. 5).

In order to compare these with references profiles, collection of blood samples from athletes for DNA profiling was divided in three rounds. On the first round of testing, 9 athletes from Nation A were controlled at a training camp. After comparison, DNA profiles of 2 athletes among the 9 could not be distinguished from the trace DNA profiles, considering the 10 corresponding loci. On the second round, 3 athletes from Nation A were tested and one of the DNA profiles obtained was undistinguishable from a third trace DNA profile. Finally, the third round concerned 18 athletes of Nation A and 5 DNA profiles of these athletes could not been distinguished from the last 5 traces DNA profiles related to this doping case.

Considering these results, a Bayesian approach was used to evaluate the statistical probability of the evidence. The DNA evidence is assessed with a likelihood ratio (LR) [9]. This metric estimates the probability of a DNA match under two alternative hypotheses that are:

- H_1: the DNA profile originated from the suspect.
- H_2: the DNA profile originated from an unknown person unrelated to the suspect.

The value of the likelihood ratio is defined by an equation representing the probability (Pr) of the DNA evidence (E) given Hypothesis 1 (H_1) or Hypothesis 2 (H_2):

$$LR = \frac{Pr(E|H_1)}{Pr(E|H_2)} \quad (1)$$

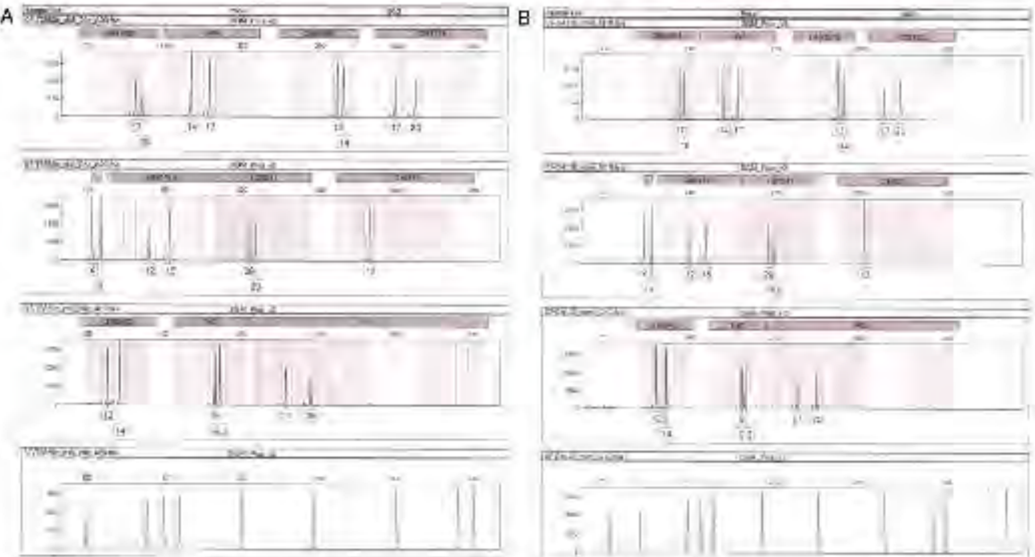

Fig. 5. DNA profile from residues found in a transfusion system (A) and a reference blood sample (B).

So to translate this ratio for a better understanding in court, a verbal scale was developed by Evett to express the weight of the evidence (Table 1) [10].

In this case study, LR values larger than 1 billion were reported for the 8 DNA matches observed. These values represent an adequate and conservative way of expressing the draw of the evidence when 10 loci SGM Plus DNA profiles are concerned [11]. On a verbal scale, DNA analyses provided an extremely strong support to the hypothesis, according to which eight suspected athletes were at the origin of the 8 matching blood stains.

Noteworthy, DNA analyses could be differentiated from anti-doping analyses since a DNA profile by itself is useless. Indeed, a reference is always needed. In other words, while the presence of a doping substance in the athlete biological fluids can prove doping, a DNA profile is only useful when it can be compared to reference material. In a forensic case, the profile would have been compared to a database. However, in the anti-doping field, DNA analyses are still not widespread and no databases have been built. An idea would be to introduce the DNA profile in the biological passport to provide a nearly exhaustive database useful to solve such cases. As it is still only a proposition, it was necessary to use other sources of information as in the forensic field, namely the contextual information and physical evidence left behind by the users. In this case, such information was really crucial to reduce the circle of suspected athletes and target the cheaters.

Table 1
Verbal scale representing the support to H_1.

LR	Verbal scale
>1 to 10	Limited evidence to support
10 to 100	Moderate evidence to support
100 to 1000	Moderately strong evidence to support
1000 to 10,000	Strong evidence to support
>10,000	Very strong evidence to support

Also, the question of eligibility of DNA analyses in the anti-doping field has never been discussed before. According to Swiss law, agreement of the athletes themselves or an order by a magistrate to conduct this type of analyses would be required [12]. In the context of the case, no magistrate could order the expertise and the athletes would certainly not have given their approval. However, in the world of professional sport, all athletes have to sign a contract, namely an Athlete's Commitment form, including several obligatory points. Actually, the FISA's form includes that: "*the athlete is willing to submit to ANY tests (blood, urine, gas, etc.) carried out by FISA*" [13]. Therefore, the Federation, on the advice of FISA head doctor, decided that it was possible to conduct tests without seeking specific consent.

As there was just a presumption against the athletes of the Nation A team, it was decided to perform a doping control only on several athletes in order to ascertain whether or not the evidence was pointing at the right athletes' group. As results showed similarities between some samples of controls and profiles obtained on the residues found in the infusions material, further investigations were carried out to draw links between the traces' DNA profiles and references coming from the suspected athletes. Based on these results and the positive DNA matches, FISA concluded that 8 rowers had violated anti-doping rules.

A last aspect of the investigation was to ascertain if the team doctor was aware of and involved in administration of the intravenous infusions. Indeed, Article 2.8 of the World Anti-Doping Code also prohibits assisting, encouraging, aiding, abetting, covering up or any other type of complicity [1]. Since the rowers claimed that they obtained and used the equipment themselves, the team doctor seemed not to be implicated. However, in a subsequent testimony, he admitted his implication after the National Federation officials recognised the doping offence. If not, the presence of fingerprints of the doctor on the intravenous equipment may have been investigated. Nevertheless, it would require a lot of work to implement fingerprints techniques and education of the Doping Control Officer on sampling and

preservation of evidence with such materials. In this case, the persons who were in contact with the materials did not take particular care during the collection. It would be interesting to evaluate the possibility of using this approach in the context of anti-doping, while keeping in mind that, as with DNA, no database is available for fingerprints. Alternatively, it would have been possible to take samples from the outside of the perfusion system and drug packages in order to look for the DNA of the epithelial left by the person who touched this material. Once again, special care is necessary to avoid contamination and the success of these DNA analyses are not guaranteed, due to environmental conditions.

Following this case, several other investigations were conducted using DNA analyses in an anti-doping context. A huge number of urine samples were compared with other samples coming from the same athletes to highlight the practice of giving negative urine hidden in pockets as reported in some countries. Another famous case which required DNA analysis was the "Puerto case" where several professional cyclists admitted blood withdrawal in order to inject it later through transfusion. These cyclists were all sanctioned. However, an unidentified blood pocket remained and DNA analysis established a positive correlation between plasma DNA present in this pocket and DNA of Alejandro Valverde. Also, it was demonstrated that this blood pocket contained EPO. The Court of Arbitration (CAS) decided to ban him for two years from all sports competitions [14].

Since that case, FISA and two other International Federations (the International Cycling Union (UCI) and the International Gymnastics Federation (FIG)) have collaborated to settle the, so called, "No Needle Policy". The use of needle must be medically justified, appropriate, administered by a certified medical professional, declared to the competition doctor and the disposal of used needles shall be conform to recognized safety. The purpose of this policy is to prevent the culture of the injection. Athletes become accustomed to this method and it may be the beginning of a gradual shift toward doping habits.

5. Conclusion and perspective

Through forensic investigation, in particular DNA analysis, FISA authorities were able to establish that 8 rowers were involved in this doping case. The analysis of the equipment provided evidence on the use of a prohibited method. Given the number of athletes implicated and the conflicting explanation from the Nation A Federation, the FISA hearing panel decided to ban not only the eight rowers for all competitions during two years but also the coaches and officials of the National Federation [15].

This case showed that the forensic approach might bring a new perspective to the anti-doping field, especially with the support of DNA analyses. Other forensic areas such as fingerprints might also provide some crucial information which, combined with traditional detection methods, would enforce evidence by linking a person with an object like a prohibited drug bottle or packaging. The use of criminal analysis could also allow identification of networks of organised doping and highlight athletes who might be connected with this activity. This will necessarily go through awareness and education of the Doping Control Officer. The International Federations should also pay special attention to the possibility of using these techniques to provide additional evidence in cases where there are still doubts about a doping offence.

The legitimacy of DNA tests in anti-doping control should also be discussed, for example with the publication of a DNA testing policy which could include guidelines for DNA analyses, authorising the use of DNA profiling in order to prove a form of doping. As a further perspective, this document might be included in the World Anti-Doping Code.

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Consensus statement

Time for change: a roadmap to guide the implementation of the World Anti-Doping Code 2015

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ABSTRACT
A medical and scientific multidisciplinary consensus meeting was held from 29 to 30 November 2013 on Anti-Doping in Sport at the Home of FIFA in Zurich, Switzerland, to create a roadmap for the implementation of the 2015 World Anti-Doping Code. The consensus statement and accompanying papers set out the priorities for the antidoping community in research, science and medicine. The participants achieved consensus on a strategy for the implementation of the 2015 World Anti-Doping Code. Key components of this strategy include: (1) sport-specific risk assessment, (2) prevalence measurement, (3) sport-specific test distribution plans, (4) storage and reanalysis, (5) analytical challenges, (6) forensic intelligence, (7) psychological approach to optimise the most deterrent effect, (8) the Athlete Biological Passport (ABP) and confounding factors, (9) data management system (Anti-Doping Administration & Management System (ADAMS), (10) education, (11) research needs and necessary advances, (12) inadvertent doping and (13) management and ethics: biological data. True implementation of the 2015 World Anti-Doping Code will depend largely on the ability to align thinking around these core concepts and strategies. FIFA, jointly with all other engaged International Federations of sports (Ifs), the International Olympic Committee (IOC) and World Anti-Doping Agency (WADA), are ideally placed to lead transformational change with the unwavering support of the wider antidoping community. The outcome of the consensus meeting was the creation of the ad hoc Working Group charged with the responsibility of moving this agenda forward.

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INTRODUCTION

A medical and scientific multidisciplinary consensus meeting was held from 29 to 30 November 2013 on Anti-Doping in Sport at the Home of FIFA in Zurich, Switzerland, to create a roadmap for the implementation of the 2015 World Anti-Doping Code. The papers delivered at the consensus meeting are published in this themed edition of the *BJSM* along with the consensus statement. The consensus statement and accompanying papers set out the priorities for the antidoping community in research, science and medicine. This consensus is timed to coincide with the publication of the 2015 World Anti-Doping Code and brings together the latest scientific and medical evidence and reaffirms the commitment of science and medicine in the fight against doping in sport.

The fight against doping in sport was formally started by the International Olympic Committee (IOC) in the 1960s, culminating in the creation of World Anti-Doping Agency (WADA) in 1999. The antidoping movement is now poised to take a major step forward in the fight against doping in sport by implementing the new 2015 World Anti-Doping Code and drawing on the major advances in science and medicine, much of which are underpinned by research funded by WADA and the International Federations of sports (IFs). As such, evidence-based, targeted, sport-specific and situation-specific strategies along with more effective analysis and improved efficiency and intelligence are approaches envisaged to lead to better deterrence of doping in sport. These strategies, combined with education and the commitment of all antidoping organisations (ADOs) to implement evidence-based programmes, should help protect the integrity of sport and all those athletes who do not dope.

Sport-specific risk assessment

The overall strategy in the fight against doping must be based on good scientific evidence, statistical analysis reflecting the prevalence of doping cases and the monitoring of illicit substances. This assessment must be sport specific as the risk and temptation to dope and the doping strategy will depend largely on the type of sport.[1] For example, within individual sports, endurance athletes in track and field, cycling or cross-country skiing would choose different substances and methods to illicitly improve their performance, in contrast to athletes who depend primarily on strength and power, such as weight-lifters, wrestlers or athletes in certain track and field disciplines. The situation in team sports is likely to be different as results depend primarily on the collective team performance, albeit that individual athletes could still be

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tempted to dope to improve performance prospects for team selection.[2]

The risk assessment must also take into account the timing during the season when peak performance is desirable and expected. The longer the preparation period for a particular event—the so-called 'out of competition' period —that may precede cycling tours or marathon running, the greater the likelihood of eluding doping controls and the temptation to illicitly augment performance during this training period. It is prudent, therefore, that the IFs along with the organisations of athletes consider sport-specific risk assessments. Such assessments should be reappraised regularly reflecting scientific evidence and information derived from forensic intelligence. The IFs, national antidoping organisations (NADOs), doctors, scientists and paramedic personnel, together with representatives from WADA and the WADA-accredited laboratories, must design and formulate the risk assessment by sharing information with the IFs, the IOC and other major event organisations. The role of WADA in this respect is to coordinate information exchange and alert the IFs and all involved stakeholders of new information on possible doping substances, methods or approaches that could potentially help uncover doping.

Prevalence measurement

A recommended approach for defining the scope of the antidoping activity is to measure the prevalence of doping. The use of appropriate epidemiological tools and the careful interpretation of survey results with an understanding of the population examined will allow the extent of the doping problem to be assessed.[3] This can be achieved by conducting questionnaire surveys as is commonly used in social sciences. Epidemiological studies can be further enhanced by measuring putative biomarkers of doping in doping-control samples. It is essential to carry out doping prevalence studies in a population of competitors, and to appropriately interpret the data before defining and implementing a programme of longitudinal follow-up. This is particularly important because of the specificity of different sports, which can be influenced differently by doping depending on the physical and physiological characteristics required for performance enhancement.

In public health, pandemics are not distributed evenly around the globe, and environmental, social and economic factors play an important role in their distribution. The same is true in sport where doping prevalence may vary as a function of the sport and the country in which it is practiced. Research recently conducted in track and field demonstrated that the prevalence of abnormal blood profiles can vary from 3% to 48% depending on the country of origin of the athletes.[3 4] Certain sports federations, sports disciplines and even countries may fear total transparency in examining their 'doping cultures' as acknowledgement may hurt their public image. However, a transparent approach is necessary if the biological monitoring of athletes is to become an effective tool in the fight against doping.

Sport-specific test distribution plans

The 2015 World Anti-Doping Code makes specific reference to the development of test distribution plans and the necessity for thoughtful and strategic approaches to testing.[5] Such approaches will ensure that the effective, intelligent and most efficient testing strategies are adopted. Fundamental to these considerations are the identification of areas of sport that might be deemed to be high risk for doping practices. International Federations and NADOs will have particular responsibilities in this respect.

Several elements should feature prominently in the development and preparation of a sport-specific testing programme. These include consideration of the unique subcultures of sport and the degree to which doping may have been 'normalised' within such subcultures, the history of doping practices within a sport, the specific physiological demands of particular events, emerging training practices, the competitive schedule, recovery from injury, an awareness of dramatic changes in performance, an understanding of the supplement marketplace and a familiarity with what is happening 'on the street' as well as 'in the stadium'.

Continual, ongoing conversation with athletes, coaches and others in the sport community can provide an enriched understanding of the likelihood of doping behaviour and emerging problematic practices. Successful antidoping programmes of the future will embody high-quality, intelligent testing practices rather than high-quantity test volumes.

The implementation of the new World Anti-Doping Code with the emphasis on more intelligent testing affords a great opportunity for enhanced, more effective and more efficient approaches to doping control. The Athlete Biological Passport (ABP) represents a further opportunity to ensure strategic and more focused testing. Implicit in the new approaches to doping control is the necessity for strategic relationships between and among ADOs at every level. Agreements between IFs, NADOs and major event organisers addressing shared approaches to results management and testing strategies—particularly as they apply to competitors who are part of ABP programmes—will benefit the antidoping movement.

The perspectives, experiences and strategies of IFs and NADOs can be integrated so as to permit more timely and cost-efficient testing, the sharing of intelligence regarding doping practices and doping practitioners and heightened vigilance of customs and other civil authorities with regard to the importation and distribution of prohibited substances particularly at the time of major sporting events. These approaches will benefit from the developing international 'community of practice' represented by leading IFs and NADOs; the development of that community will itself be stimulated by the growth of strategic partnerships and cooperative antidoping activities.

Storage and reanalysis

New peptides or designer drugs may be used by athletes who feel that there are currently no reliable analytical tests available. However, the 2015 World Anti-Doping Code allows for the storage of samples for up to 10 years, which markedly transforms the antidoping environment. The deterrent effect of delayed testing with newly devised analytical methods is substantial. It is important that ADOs implement this process; the new 2015 International Standard on Testing and Investigations sets out the requirements for ADOs to test, store and reanalyse samples. The IFs and other ADOs must prioritise which samples should be stored from which competitions on the basis of their risk assessments. Such decisions should, to the extent permissible under applicable laws, remain confidential to optimise the deterrent and detection elements of this new approach to doping control. It is important that the storage of samples be conducted in a manner that enables future analysis with methods that may not yet be fully developed or operational. An example would be the future analysis of molecular signatures of doping (see article by Pitsiladis *et al*).[6] It is also imperative that samples are stored in a manner that protects the integrity of biological samples and the antidoping process, having due regard

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to legal requirements surrounding handling of human biological materials and related data.[5]

Analytical challenges

The continuously growing knowledge in medicine, molecular biology, biochemistry and biotechnology has substantially expanded the options to pharmacologically manipulate the performances of athletes. Unsurprisingly, this has resulted in the suspected and proven misuse of a wide range of peptide hormones and substances such as insulin, insulin-like growth factor 1, human growth hormone (hGH), epoetins, chorionic gonadotrophins, gene doping substances including RNA interference (RNAi), 'designer drugs' (eg, Tetrahydrogestrinone) as well as non-approved, emerging or discontinued compounds (eg, aminoimidazolecarboxamideriboside-AICAR, GW1516, selective androgen receptor modulators, hypoxia-inducible factor stabilisers and erythropoietin (Epo)-mimetic agents). These developments represent a considerable analytical challenge for antidoping scientists, which require dedicated research and the development of new methodologies. Concerted activities with civil authorities are also necessary to understand and combat the changing scope of doping practices and products.[7]

Analytically, the issue of new, discontinued, or 'tailored' drug entities has been successfully tackled by applying non-target/open approaches, biomarker-based assays (eg, haematological, steroidal and endocrinological modules of the ABP), 'omics' strategies and monitoring effects of drug (mis)use rather than the administered drug. This has been carried out via proactive and retrospective monitoring programmes. Some recent successes include the introduction of section 'S.0' to the WADA Prohibited List, new detection methods for the determination of RNAi-based compounds in blood and urine, the analysis of Epo microdosing, adverse analytical findings for the non-approved drugs GW1516, andarine and ostarine, as well as new methods to detect the illicit use of natural compounds such as AICAR.[7–9]

Forensic intelligence

The importance of investigations is enhanced in the 2015 World Anti-Doping Code[10] to further encourage ADOs to pursue antidoping violations based on the strongest evidence possible. While investigations as the primary means of proving doping violations are uncommon in sport, investigations have been successful in highlighting doping practices and developing novel approaches in the fight against doping in sport.

High-profile investigations include the Bay Area Laboratory Co-Operative (BALCO) investigation that was undertaken in the USA in 2003.This case resulted in the prosecution of several athletes for the use of hGH and new designer steroids and led to new laboratory detection capabilities and changes to the World Anti-Doping Code.[11 12] In 2006, a Spanish investigation code-named Operation Puerto highlighted serious doping practices involving Dr Eufemiano Fuentes and a number of elite athletes.[13 14]

In 2013, the Australian Crime Commission uncovered links between organised crime and professional sporting teams and the use of performance-enhancing 'peptides' and other illicit substances.[15] This investigation led to a team in the Australian Football League being charged and subsequently sanctioned.[16] At the heart of this case were the activities of support staff. Therefore, it follows that close scrutiny of support staff is explicit in the 2015 World Anti-Doping Code. The successful conclusion of each of these highlighted cases relied on forensic science and other modern investigative techniques now at the disposal of IFs and NADOs and in accordance with the investigation aspects of the 2015 World Anti-Doping Code.

A forensic intelligence model of antidoping investigations was proposed by Marclay *et al*[17] and included broader exploitation of information held at different levels by antidoping partners such as the police, borders agencies and postal services, strategic Internet monitoring, physical and chemical drug profiling and doping script analysis within a forensic intelligence framework. Tactical use of forensic intelligence tools relies largely on the exploitation of bioanalytical results, documents linked to doping practice and seizures of prohibited substances. At the operational level, the exploitation of such information serves to uncover trends related to the abuse of prohibited substances, the existence of organised doping programmes including the trafficking of doping agents, and helps identify their structure and mechanisms of operation. Strategic Internet monitoring also allows the identification and monitoring of online sales websites, forums, blogs, social networks and other online media, thus helping to create a clearer picture of the market and emergence of new trends.[18] Monitoring of the physical and chemical profiles of seized products further enhances the understanding of the organisational structure of the trafficking of prohibited substances.[19] Drug profiling as well as digital and other data allow the building of 'inference models' that can link to product seizures, highlight distribution networks and identify the sources of supply. Finally, script analysis can map the complete sequence of activities before, during and after doping to identify the key stages and possible intervention points where the doping process might be disrupted or even prevented.[20]

Psychological approach to optimise most deterrent effect

The perception of the likelihood of detection, the severity of the penalty and the speed with which sanctions will be applied all appear to deter doping behaviour.[21] Better understanding of doping deterrents will enhance doping control programmes and reinforce the need for testing strategies to be carefully considered, strategically applied and robustly enforced.

The sense of 'right and wrong' and the perception of normative behaviour within a sport community are perhaps the most fundamental determinants of appropriate sporting behaviour.[22–24] Doping practices have been mostly embedded in sports in which it was widely understood that such behaviours were part of the sport 'culture'. Therefore, sport organisations should consistently emphasise that drug-taking behaviour is fundamentally contrary to the principles and precepts of sport, that is, against the spirit of sport.

Sport can profoundly mould and modify attitudes and beliefs. The clear, unequivocal expression of a set of expectations regarding conduct and behaviour within a sport can have a powerful and enduring impact. The degree to which these expectations are upheld by athletes can enhance their legitimacy in the eyes of their fellow competitors and strengthen the perception that violations of such expectations are wrong. The profound disapproval that follows the violation of broadly valued standards of behaviour can be an immensely powerful sanction and the desire to avoid such disapproval an equally potent deterrent. Therefore, the creation of what has been described as a 'moral cosmology' and an associated 'moral community' is central to the development of a sporting community in which doping practices are reduced to an absolute minimal level (accepting that there will always be those who succumb in sport, as elsewhere, to the temptation to cheat).[21 25]

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The ABP and confounding factors

Typical doping control based on the direct detection of a substance or its metabolites is an effective approach. However, it has limitations particularly when an athlete may be using substances on an intermittent and/or low-dose basis, which may therefore go undetected under even the most robust In-of-Competition and Out-of-Competition Doping Control programme. Furthermore, the availability of substances virtually identical to those produced by the human body, such as the native form of Epo, testosterone and growth hormone necessitated a new drug-testing paradigm.

Longitudinal profiling, which eventually became harmonised into the scientifically robust WADA ABP programme, is a complementary and alternative means to traditional doping control. Doping leaves a characteristic 'fingerprint' on the biology of the athlete and the ABP is used to identify that fingerprint, and thus the occurrence of doping. Once a biomarker of doping is implemented in the ABP, the potential to detect those changes brought on by performance-enhancing drugs is increased; it may prove possible to detect changes caused by substances that have not yet been identified.[26]

The intelligent and timely interpretation of ABP data can lead to target testing for specific substances. Alternatively, an atypical passport finding which is confirmed by an Expert Panel can lead to an athlete being charged with an antidoping rule violation without a 'positive' test (Adverse Analytical Finding). Thus, the ABP can be seen as an innovative and reliable antidoping tool as reflected by the findings of Court of Arbitration for Sport panels in several cases. The introduction of the ABP also provides a strong doping deterrent and a boost to the credibility of the fight against doping in any given sport.[27] The ABP does not only involve the monitoring of biological markers. Confounding factors such as age, sex and exposure to higher altitude for the haematological module are also included in the passport for improved decision-making.[28] Several confounding factors are also described in the WADA 2014 endogenous anabolic steroids technical document (TDEAAS2014).[29] Detailed information regarding sample collection, transport and analysis is included in the technical documents that accompany the WADA ABP Operating Guidelines.[28]

Data management system—Anti-Doping Administration & Management System

Regardless of advancements in science and enhanced antidoping practice and policy, the fight against doping in sport can only succeed if there is a coordinated effort to ensure that the limited resources are used effectively. In this regard, the collection, analysis and sharing of doping control-related information and intelligence are imperative. Only by using a single database to collect and disseminate such information can the global antidoping community intelligently coordinate their efforts. Anti-Doping Administration & Management System (ADAMS) provided by WADA, adapts to support the ever-changing antidoping environment. A single database also ensures consistency in protecting the rights of athletes vis-à-vis their information and ever-emerging data protection best practices.

Education

One of the objectives of successful antidoping education is to ensure that all those involved in sport understand the harm caused by doping to the health of athletes and to the integrity and essence of sport. As all sport-related stakeholders have a role to play to promote clean sport, educational efforts must be inclusive of the broad sporting community including athletes, coaches, physicians, teachers and parents. This objective will require the commitment of the IOC, IFs, governments (to reach schools and community-level sport) and NADOs, with WADA as the coordinator.

Education should be ongoing and sustained; it must take place throughout the entire sporting career of an individual and focus on values and good decision-making skills as well as an appreciation of the roles and responsibilities of athletes. The education of athletes has to start as early as possible, preferably prior to an athlete's first national/international competition. For example, FIFA introduced a standard educational programme to all participating teams of the FIFA U-17 World Cup (boys and girls) 2012 that was overseen by doctors responsible for the competition. This is in addition to other grassroot education programmes already implemented by organisations such as UEFA that involve the education of more than 1000 young international football players each year. For the success of these programmes, the message presented to athletes needs to be clear and at the correct level. The use of posters, to decorate the typically 'unfriendly' surroundings, and advice cards could also facilitate such communication.

Research needs and necessary advances

Approaches to detect doping have improved significantly in recent years but remain imperfect and therefore new direct and indirect detection methods are required. New integrative 'Omics'-based solutions are being developed that have the potential to improve the analytical performance of current detection methods.[30] In particular, WADA is funding studies to identify a 'molecular signature' of recombinant human erythropoietin (RhumanEpo) doping and preliminary results are promising.[31] For example, in the first systematic study to be conducted, the expression of hundreds of genes were found to be altered by RhumanEpo with numerous gene transcripts being differentially expressed after the first injection and further transcripts profoundly upregulated during and subsequently downregulated up to 4 weeks postadministration of the drug, with the same transcriptomic pattern observed in all participants. The identification of a blood 'molecular signature' of RhumanEpo administration is the strongest evidence to date that gene biomarkers have the potential to substantially improve the analytical performance of current antidoping methods such as the ABP for RhumanEpo detection. These encouraging results serve to strongly reinforce the feasibility and need for this complex, expensive and technically demanding approach involving leading industry partners for the detection of banned substances and methods. Therefore, research using an 'omics'-based approach involving genomics, transcriptomics, proteomics and metabolomics should be greatly intensified in order to achieve improved detection of Recombinant human Epo and other doping substances and methods difficult to detect such as growth hormone and blood transfusions.

Inadvertent doping

A major objective of the global fight against doping is the protection of the clean athlete, and hence the need to inform athletes of the risks of inadvertent doping. In recent years, antidoping research has identified contaminated nutritional supplements and food as the principal sources of inadvertent doping. Nutritional supplements have been contaminated with various stimulants, β2-agonists, prohormones, 'classic' anabolic steroids and non-approved designer steroids.[32 33]

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Similarly, the consumption of certain foods, especially meat products, is of particular concern as they may result in an adverse analytical finding. Recent investigations have shown that the anabolic agent clenbuterol is misused in some countries as a growth promoter in cattle feeding. The consumption of meat from clenbuterol-treated cattle may lead to adverse analytical findings and to poisoning cases.[34 35] WADA has communicated with specific governments to address this concern as it relates to doping and sport. WADA and FIFA are also working on studies that may differentiate the source of clenbuterol to detect whether the substance found in the body may be due to an indirect ingestion from an animal product. First promising results have already been obtained.[36 37] The identification of such 'doping traps' and the dissemination of the knowledge of such sources of inadvertent doping to all stakeholders are important aspects in the protection of athletes.

Management and ethics: biological data

The introduction of the ABP is a reason to consider expanding the role of the medical profession in the fight against doping in sport. The ABP is a recently validated approach to the identification and prosecution of doping rule violations. The routine implementation of the ABP may result in the inadvertent identification of potential clinical situations and this will need to be addressed by medical experts. When alerted to laboratory findings, physicians are obliged to inform the athlete via the ADOs if there is a suspected pathology. ADOs, particularly the IFs and the NADOs, must respond accordingly to ensure the appropriate involvement of medical professionals in the process of individual case management. At present, the mandate of ADOs is primarily to ensure that the health of the athlete is not affected by entering a doping spiral rather than as a health check system, and the process typically does not involve a physician. New rules would be required to address this issue.[5]

Whenever doctors affiliated with ADOs are involved in results management, they must ensure that anomalous results of potential clinical significance are investigated appropriately. In such situations, communication with other physicians involved in the care of the particular athlete outlining the findings, their implication and suggesting, when necessary, an approach to their further investigation has been a common approach. It is unreasonable to expect that non-physicians would have the training, experience or perspective to assume responsibility for such a process. The management of such cases requires distinct medical knowledge and clinical experience.

The introduction of the ABP has raised a number of questions that reflect a profound concern for issues of confidentiality and the responsibility of those in receipt of biological information to take action if and when information that may relate to the health of a competitor becomes apparent. There may be significant national considerations to take into account in such circumstances. In some jurisdictions, testing authorities and laboratories are not considered or accredited as providing healthcare-related services and the disclosure of information emanating from such facilities, subsequently used for clinical purposes, may jeopardise such accreditation. Clearly, there is no expectation that analytical laboratories should see their role as expanding into 'clinicopathological' domains. The identification of anomalous findings by a clinician should be seen as an *en passant* phenomenon occasioned by a clinician's review of antidoping laboratory results and prompting further clinical-standard investigations. The activities of clinicians in this respect should not be misinterpreted as evidence that antidoping analyses reflect clinical activities on the part of the antidoping laboratory.

However, clinicians would argue that they have a fundamental ethical responsibility to take action when provided with information that may reflect an underlying pathological condition. Physicians are obliged to alert the ADOs if they note anomalous results that are suspicious of pathology. The assessment of results by laboratory and medical experts (as part of the ABP) is carried out anonymously, therefore contact must be made via the ADO. Notably, in urine samples from male athletes, an elevation of the levels of human choriogonadotropin hormone (hCG) is quite common with approximately 90 cases a year according to WADA statistics.[38] Elevated hCG may be due to the intake of the exogenous hormone but could reflect an underlying pathology—most typically testicular cancer. For all such cases, a specialised medical examination must be recommended as soon as possible to ensure appropriate investigation and treatment. Physicians experienced in providing oversight to antidoping programmes are familiar with this scenario and understand the importance of their intervention to ensure that proper clinical attention is given to the athletes concerned. WADA has provided clear instructions to ADOs to contact athletes to seek further medical investigation when elevated hCG levels are detected. There are a number of documented cases where such early intervention has led to a complete cure of the underlying condition and can be seen as an extremely positive aspect of doping control activities. hCG testing is part of routine analytical doping tests and not part of the ABP.

Equally challenging is the question of the right of competitors to have access to their test results. This is a complex and highly problematic issue given that such access may allow doping competitors to manipulate or modify their strategies so as to be more likely to escape detection. Furthermore, national legislation may, in many jurisdictions, mandate the release of such information; the question of the timing of such release may be critically important in ensuring the integrity of the testing system. These challenges notwithstanding, the importance to protect the data accumulated in the conduct of doping control programmes is paramount. It is important for sport organisations and their officials and staff to understand the robust and rigorous approaches that are used to safeguard personal health and related biological information in other community settings.

Summary and conclusions

The purpose of this paper was to summarise the results of the consensus meeting on Anti-Doping in Sport sponsored by FIFA. The participants achieved consensus on a strategy for the implementation of the 2015 World Anti-Doping Code. Key components of this strategy include: (1) sport-specific risk assessment, (2) prevalence measurement, (3) sport-specific test distribution plans, (4) storage and reanalysis, (5) analytical challenges, (6) forensic intelligence, (7) psychological approach to optimise most deterrent effect, (8) ABP and confounding factors, (9) data management system (ADAMS), (10) education, (11) research needs and necessary advances, (12) inadvertent doping and (13) management and ethics: biological data. True implementation of the 2015 World Anti-Doping Code will depend largely on the ability to align thinking around these core concepts and strategies. FIFA, jointly with all other engaged IFs, the IOC and WADA are ideally placed to lead transformational change with the unwavering support of the wider antidoping community. The outcome of the consensus meeting was the creation of the ad hoc Working Group charged with the responsibility of moving this agenda forward.

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Analysis

A forensic perspective of the AFL investigation into peptides: an antidoping investigation case study

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ABSTRACT

Background The World Anti-Doping Agency (WADA) is introducing enhancements to doping investigations in its 2015 Code, which include improved sharing of information between antidoping organisations (including sporting bodies) and enhanced accountability of athlete support staff. These additions will improve the control of links between sports doping and organised crime. In February 2013 the Australian Crime Commission released a report that linked several professional sporting codes, professional athletes with links to organised crime, performance enhancing drugs and illicit substances. Following this report the Australian Football League (AFL) partnered the Australian national antidoping organisation to investigate peptide use in Australian football.

Methods This review compared the model proposed by Marclay, a hypothetical model for anti-doping investigations that proposed a forensic intelligence and analysis approach, to use the forensic capabilities of the AFL investigation to test the model's relevance to an actual case.

Results The investigation uncovered the use of peptides used to enhance athlete performance. The AFL investigation found a high risk of doping where athlete support staff existed in teams with weak corporate governance controls. A further finding included the need for the investigation to provide a timely response in professional team sports that were sensitive to the competition timing. In the case of the AFL the team was sanctioned prior to the finals as an interim outcome for allowing the risk of use of performance-enhancing substances. Doping violation charges are still being considered.

Discussion Antidoping strategies should include the investigation of corporate officers in team doping circumstances, the mandatory recording of all athlete substance use during competition and training phases, the wider sharing of forensic intelligence with non-sporting bodies particularly law enforcement and collaboration between antidoping and sporting organisations in doping investigations.

Conclusions The AFL investigation illustrated the importance of the 2015 WADA Code changes and highlighted the need for a systematic use of broad forensic intelligence activities in the investigation of doping violations.

INTRODUCTION

The World Anti-Doping Agency (WADA) in partnership with its sport and government stakeholders ratified the 2015 WADA Code (WADC) in November 2013. Three of the five key theme changes to the Code were the enhancement of doping investigations as a means of supporting doping violations, greater cooperation between antidoping organisations including investigations, and greater accountability of athlete support staff.[1]

The 2015 WADC allows for investigations by antidoping organisations (ADO) to utilise information other than athlete test results where there may have been doping violations. This information includes forensic intelligence and evidence gathered in a doping violation investigation. The 2015 WADC promotes collaboration between all agencies responsible for the fight against doping, including investigating the involvement of athlete support staff. To achieve these aspirations new capabilities will be required of ADO.

In April 2013, Marclay *et al*[2] published a hypothetical model for antidoping investigations that proposed an integrated use of forensic intelligence and analysis. In this paper the authors reviewed the Bay Area Laboratory Co-operative (BALCO) and Operation Puerto investigations and discussed the relatively rare nature of forensic antidoping investigations.

In both these investigations sharing of information on suspicious activities between antidoping authorities and law enforcement agencies proved instrumental to the detection of organised doping networks, the collection of further evidence and ultimately successful prosecution. The investigation of the BALCO case was a catalyst for the recognition of the value of non-analytical sources of information for identifying and proving a doping violation in the absence of adverse analytical findings. Evidence collected on athletes and athlete support staff included seizures of prohibited substances, documents and digital data.

In February 2013 the AFL and Australian Sports Anti-Doping Agency (ASADA), the Australian National Anti-Doping Organisation, started an investigation into peptide use at three AFL clubs by using modern investigative techniques.[3] This investigation took 7 months and involved nine investigators and several technical experts.

This review is a case study that explores the role of the forensic intelligence approach of the AFL peptides doping investigation with particular reference to the Marclay theoretical model. The paper demonstrates that this type of doping investigation can include corporate governance issues, the central role of athlete support staff and the need for a timely response in a professional team sport which has competition time pressures.

AUSTRALIAN FOOTBALL LEAGUE

Australian football is an indigenous football code that is a physical, body contact sport, with physiological demands that involve aerobic capacity,

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strength, power and ball handling skills.[4 5] The AFL competition is conducted as a professional league of 18 teams with over 810 active players.[6] The age of players ranges from 18 to 38 years and all players are bound by a standard contract with obligations to their team and the AFL. The revenue of the AFL in 2012 was $A425 million. The operating budgets of the 18 teams varies from $A35 million per annum to $A75 million pa and the club supporter bases, effectively the shareholders of each team, varies from 35 000 to 80 000 members. The income of players varies from $A80 000 per annum to $A1.2 million per annum.

Based on the physiological demands of the game and the current knowledge of global doping practices, there is a substantial risk of AFL players and teams cheating with anabolic androgenic steroids, hGH, EPO and blood doping. To combat this risk the AFL has a WADA compliant antidoping code and robust testing platform including blood and urine samples.

AFL INTEGRITY UNIT

The AFL established a well-resourced Integrity Unit in 2008 in a global environment where corruption, match fixing, improper betting, growing use of performance enhancing drugs (PED) and illicit substances (IS) was occurring alongside the growth of organised crime involvement in some sports.[6] The Unit is responsible for AFL policy, intelligence and investigations of issues affecting the integrity of the competition such as match fixing, doping, illicit drugs, salary cap cheating, the draft and player transfer system. The unit educates players on corruption and doping risks, shares information with betting agencies, collaborates with ASADA on antidoping strategy, tests for new and emerging PED risks (overseas testing, blood and steroid profiling, hGH, EPO and CERA), tests for IS out-of-competition, and investigates off-field athlete behaviour incidents. The unit analyses all this information to inform the AFL on existing and arising issues across all areas of interest.

At the start of the peptides investigation the AFL Integrity Unit had 11 full or part time employees, including a manager, one investigator, one intelligence analyst and a sports physician. During the course of the investigation the AFL and ASADA employed a number of additional staff—investigators, analysts, forensic data experts, pharmacologists and specialist endocrinologists. The Integrity Unit has subsequently been expanded to 14 staff (figure 1).

AFL-ASADA INVESTIGATION

In February 2013 the Australian Crime Commission released a report that identified several professional sporting codes and a number of professional athletes with links to organised crime, as well as use of PED and IS.[7] Following the report's release the AFL and ASADA partnered to undertake an extensive investigation into the use of the peptides and other substances in the AFL.

As in Marclay *et al*'s[2] theoretical model of forensic intelligence, the AFL and ASADA emphasised collaboration in a multidisciplinary environment to improve investigative efficiency, avoid fragmentation of information and share specialist resources. This investigation was focused and centralised, with complementary forensic activities and scientific resourcing that allowed the exchange of information and sharing of expertise.

Owing to the contract arrangements binding players and officials to a code of practice, the AFL had extensive and, at the time, superior investigative powers to that of ASADA, which facilitated the inquiry and substantially assisted both parties. This collaboration is consistent with one of the new directions of the 2015 WADC.[1]

The AFL investigative powers included access to; players and officials for interviews, mobile phone records of players and officials, emails, team servers, laptops and team files including financial records (table 1). The information obtained was cross-referenced and analysed using internal investigators and intelligence analysts and an external forensic accounting team. As with the BALCO investigation the financial records were of particular value.

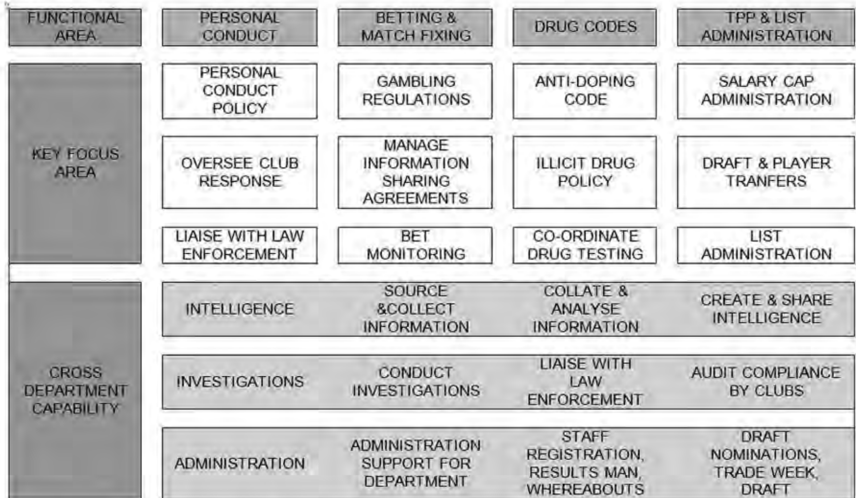

Figure 1 Illustrates the various functions and work areas of the Integrity Unit.

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Table 1 Summary of information and materials collected and analysed

Data retrieved	Number
Investigations personnel	25
Interviews	130
Duration of interviews	600+h
Digital record seizures	1.5 terabytes
Mobile phone seizures	8
Text messages/call content	98 000
Laptop computer seizures	4
Emails	449 gigabytes, 16 million emails
Line entries in financial systems	6.8 million
Drug test and steroid profiles	29
Drug test declarations reviewed	61
AFL cost	$A1.3 million

AFL, Australian Football League.

The information was obtained from a wide range of people and places and was stored in the AFL Integrity Unit's case management and intelligence database. This sophisticated database permitted the collation and analysis of large amounts of data by intelligence analysts and investigators which permitted the identification and visualisation of the key elements and underlying mechanisms of the possible doping activity under scrutiny. Linking information from different sources provided a comprehensive picture to examine the interactions between athletes and support staff engaging in potential doping. The AFL-ASADA investigation was consistent with the tactical use of forensic intelligence as proposed by Marclay *et al*.[2]

As reported by the Australian Crime Commission and partly confirmed by the investigation,[3 7–9] the substances supplied to athletes by team support staff were loosely labelled 'peptides' and 'amino acids' by the perpetrators. The substances named in the ACC report included CJC 1295, GHRP-6, GHRP-2, AOD 9604, hexarelin, ipamorelin, semorelin and other substances, some of unknown nature. The mix of these peptides varied from team to team and athlete to athlete. Generally the substances were given in injection form by non-medical trained team support personnel. In the AFL investigation, due to the lack of individual athlete and team documentation, the precise nature and doses of substances that were given to each athlete was uncertain or unknown.

Under the WADA Prohibited List many of these substances are prohibited, being human growth hormone (hGH) releasing substances or sections of the hGH molecule. They are listed on the WADA Prohibited List under categories S2 (peptide hormones, growth factors and related substances) and/or S0 (non-approved substances).[10]

Immediately following the AFL investigation one team was charged with 'bringing the game into disrepute' under AFL Rules.[8 9] The team was fined $A2 million, sanctioned with loss of player draft picks and not permitted to compete in the 2013 championship finals. By accepting the sanctions, the team acknowledged there had been a risk its players had been administered WADA-prohibited substances, the basis of the initial charges. Three team support staff officials were also sanctioned with suspension or fine.

The impending 2013 AFL championship finals highlighted the need for the League to promptly respond to potential team-based doping, in contrast to the longer legal process for determining whether team or individual doping violations had actually occurred. The team's elimination from the championship finals avoided the possibility of athletes who had potentially used banned substances contributing to a team's success in finals. The determination of doping violations for individuals and/or the teams is a complex legal exercise and, at the time of writing this paper, is under consideration by ASADA.

In parallel with the AFL investigation one of the clubs undertook an internal review.[11] The report described 'a pharmacologically experimental environment never adequately controlled or documented', 'the use of exotic supplements', 'marginalisation of traditional medical staff' and the failure of good governance.[11] The report found that uncontrolled athlete support staff caused by a breakdown of normal organisation controls, lack of documentation and poor staff accountability led to the potential doping activities. The report recommended a number of internal governance reforms. The AFL-ASADA investigation confirmed the corporate governance failure.

It would appear that a doping investigation of a team requires an assessment of corporate governance practices. Additional external regulator involvement such as those overseeing healthcare funding, occupational health and safety and corporation law may also be necessary. Such investigations may involve a number of regulators and not be exclusively confined to antidoping organisations. Equally peak sporting bodies need to expand their own roles beyond that of sporting competition regulators.

The AFL-ASADA investigation was resource intensive from financial, technical and personnel perspectives. This raises the question of who should be accountable for the cost of such investigations as it is likely National Anti-Doping Organisations may be reluctant to take on the full liability of such activities.

DISCUSSION

To our knowledge the depth and range of forensic intelligence had not previously been used in an antidoping investigation. Collating information of potential doping activities in a database the investigators were able to better understand the possible operations of the supply network. Intelligence produced through extensive analysis of data allowed the investigation team to identify the structure and dynamics of the illicit activities. The AFL-ASADA investigation demonstrated the need for, and efficiency of, a partnership between sporting bodies and antidoping organisations. This inquiry highlighted a tactical, and to some extent operational, use of the Marclay *et al*[2] model. Data gathering and processing detected irregular activities and their evolution, and determined the role of athletes and support staff, providing evidence for potential court proceedings. As such, the investigation illustrated the practicality of Marclay *et al*'s model.

While the investigation was largely reactive in nature, early detection and a more timely response to future potential doping activities should now result. The exchange of information between the AFL and ASADA, and the potential additional involvement of law enforcement, may prevent similar breaches in the future.

Governmental agencies such as the Australian Crime Commission, Australian Customs, Australian Federal Police and State law enforcement hold extensive information relevant to the fight against doping. Prohibited substances entering the Australian territory or already on Australian soil are regularly seized by law enforcement agencies, informing antidoping organisations on substance availability on the black market and their distribution. The trafficking of PED and IS often originates from the same criminal organisations[7] and evidence on doping

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activity is likely to appear in law enforcement files. Systematic sharing of this type of information with the sporting organisations, such as the AFL, and NDOs, such as ASADA, should enhance the capacity to detect organised doping in sport. Resources, costs and reaction time would be reduced through partnership with law enforcement, with their superior investigative skills and experience with criminal organisations.

Early detection through strategic internet monitoring of PED should identify new trends in the structure of the doping market. The analysis of digital data from sales websites, forums, blogs, social networks and other online media should contribute to greater knowledge of distribution network activity, raise prevention alerts to be passed on to athletes and support staff, as well as identification of individuals who are actively engaged in doping practices. Indeed this case highlighted the emergence of peptides into the doping marketplace and their access through antiageing clinics and compound pharmacies. The use of compounding pharmacy to by-pass national regulation, the advisory role of personnel in antiageing clinics and their promotion of non-scientific practices such as peptide use were identified by the investigation. The WADA status of some of these products was unclear and an early detection strategy would allow greater clarity of the status of such new substances.

The AFL case study highlighted time and operational restraints of the competition itself. The investigation was undertaken alongside weekly competition and could have disrupted a team's ability to function. Furthermore, the AFL-ASADA investigation led into the championship finals period that raised the practical issue of participation of a team potentially advantaged by the use of PEDs. The AFL overcame the issue of timeliness of action by using code of conduct clauses in its rules and based its actions on the significant risk of doping as opposed to a proven doping violation.

Finally, the peptides use in the team was overseen by team support staff. Good corporate governance practices in sporting teams is essential and this investigation questions whether team corporate officers should be made accountable in antidoping codes for governance breakdown that increase the risk of doping.

CONCLUSIONS

The 2015 WADC will enhance doping investigations, increase accountability of athlete support staff and improve sharing of antidoping intelligence and information. This AFL case study illustrates the importance of these changes and highlights the role of broad forensic intelligence activities in the investigation of doping violations. The model proposed by Marclay *et al*[2] compared favourably with this case study and that model appears to guide utilisation of the breadth forensic intelligence in doping investigations.

The findings of the AFL investigation demonstrated that poor management of athlete support staff combined with weak corporate governance controls, posed a doping risk. The investigation further verified the emergence of peptides in doping activities and the role of antiageing clinics and compound pharmacies as potential conduits of doping activities.

In professional team sports there is the need for timely management of investigation outcomes that are not easily aligned with normal legal antidoping practices. Antidoping strategy changes that could be considered include the mandatory recording of athlete substance use during competition and training phases, the wider sharing of forensic intelligence with non-sporting bodies particularly law enforcement, the potential role of an examination of corporate governance practices within a sporting organisation and further improved collaboration between antidoping and sporting organisations in doping investigations.

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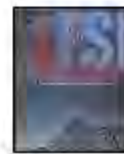

A one-year monitoring of nicotine use in sport: Frontier between potential performance enhancement and addiction issues☆

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ABSTRACT

Tobacco consumption is a global epidemic responsible for a vast burden of disease. With pharmacological properties sought-after by consumers and responsible for addiction issues, nicotine is the main reason of this phenomenon. Accordingly, smokeless tobacco products are of growing popularity in sport owing to potential performance enhancing properties and absence of adverse effects on the respiratory system. Nevertheless, nicotine does not appear on the 2011 World Anti-Doping Agency (WADA) Prohibited List or Monitoring Program by lack of a comprehensive large-scale prevalence survey. Thus, this work describes a one-year monitoring study on urine specimens from professional athletes of different disciplines covering 2010 and 2011. A method for the detection and quantification of nicotine, its major metabolites (cotinine, *trans*-3-hydroxycotinine, nicotine-N′-oxide and cotinine-N-oxide) and minor tobacco alkaloids (anabasine, anatabine and nornicotine) was developed, relying on ultra-high pressure liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC–TQ-MS/MS). A simple and fast dilute-and-shoot sample treatment was performed, followed by hydrophilic interaction chromatography–tandem mass spectrometry (HILIC-MS/MS) operated in positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) data acquisition. After method validation, assessing the prevalence of nicotine consumption in sport involved analysis of 2185 urine samples, accounting for 43 different sports. Concentrations distribution of major nicotine metabolites, minor nicotine metabolites and tobacco alkaloids ranged from 10 (LLOQ) to 32,223, 6670 and 538 ng/mL, respectively. Compounds of interest were detected in trace levels in 23.0% of urine specimens, with concentration levels corresponding to an exposure within the last three days for 18.3% of samples. Likewise, hypothesizing conservative concentration limits for active nicotine consumption prior and/or during sport practice (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-N′-oxide, cotinine-N-oxide, anabasine, anatabine and nornicotine) revealed a prevalence of 15.3% amongst athletes. While this number may appear lower than the worldwide smoking prevalence of around 25%, focusing the study on selected sports highlighted more alarming findings. Indeed, active nicotine consumption in ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics was found to range between 19.0 and 55.6%. Therefore, considering the adverse effects of smoking on the respiratory tract and numerous health threats detrimental to sport practice at top level, likelihood of smokeless tobacco consumption for performance enhancement is greatly supported.

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1. Introduction

Origins of tobacco consumption date back to South American Aztec shamanic rites. Little could predict this ancient tradition would turn into a world epidemic due to nicotine, the principal alkaloid found in tobacco leaves where it acts as a natural insecticide.

Indeed, this molecule exhibits a variety of pharmacological properties sought-after by consumers and responsible for persistent addiction issues. Amongst the numerous neurotransmitters released in the central nervous system by stimulation of nicotinic cholinergic receptors, dopamine is associated with rewarding experiences [1]. Promotion of related positive reinforcing effects results in vigilance and cognitive function enhancement together with relaxation, reduced stress, mood modulation and lower body weight [2,3]. Interestingly, nicotine also triggers a significant increase of pulse rate, blood pressure, blood sugar and epinephrine

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release owing to simultaneous stimulant and relaxant properties [4,5]. As a consequence, nicotine addiction develops through repeated exposure to experience positive reinforcing effects with relief of withdrawal symptoms [1].

Despite public knowledge on toxicity and carcinogenic properties of tobacco smoke components, worldwide consumption is responsible for over 5 million deaths each year, a number expected to grow up to 8.3 million by 2030 [6]. Noteworthy, tobacco use extensively contributes to the burden of disease from the four leading causes of death, including ischaemic heart disease, cerebrovascular disease, infections of the lower respiratory system and chronic obstructive pulmonary disease [7]. In addition, trachea, bronchus and lung cancers mortality is an additional major health issue significantly correlated with tobacco consumption.

Therefore, treatment of nicotine addiction is a global concern which is considered from different perspectives, supporting either smoke cessation programs or preventive and harm-reducing oriented smoke regulation policies. Indeed, current strategies focus on different aspects of the problem, including various approaches such as medical advice to pharmacotherapy, or tax increases to implementation of smoke-free environments and distribution of educational material on tobacco consumption [6,8]. At present, prohibition of smoking in public places is growing popular worldwide, proving efficient in the intent of reducing both first- and second-hand exposure while helping smokers to quit. As a consequence, the tobacco industry is in the need of marketing a diversified range of products to bypass such a restrictive legislation which is obviously detrimental to the business. Accordingly, a solid attempt to advertise smokeless tobacco products, in particular snus, is observed throughout Europe and North America. However, equivalent addictive properties have been observed when comparing smoked and smokeless nicotine, which is a serious promoting factor for smoking initiation [9]. In addition, despite avoiding health issues associated to tobacco smoke, consumption of smokeless tobacco may be responsible for oral, esophageal and pancreatic cancers due to the presence of over 28 carcinogenic constituents, but also heart diseases and lesions of the oral tissues [10,11].

Therefore, smokeless tobacco is a very attractive drug from a doping perspective, considering the performance enhancement pharmacological properties of nicotine and the absence of direct adverse effects on the respiratory tract [12]. Actually, recent observations on nicotine consumption depict this phenomenon as a popular trend in winter sports, regardless of the level of competition [13–15]. In particular, a recent study on the 2009 Ice Hockey World Championships brought alarming findings as active nicotine consumption before or/and during the games was highlighted for about half of the athletes [16]. Nevertheless, nicotine still does not appear on the 2011 World Anti-Doping Agency (WADA) Prohibited List or Monitoring Program, despite satisfying the three inclusion criteria [17,18]. Indeed, this substance may enhance sport performance, represents a potential health threat for the athlete and may also alter the spirit of sport due to the negative image associated with tobacco consumption. However, prevalence of nicotine consumption in sport still suffers from a lack of large-scale comprehensive survey.

Thus, this work describes a one-year monitoring study on nicotine use in a broad range of sports between 2010 and 2011. Accordingly, presence of nicotine, its four main metabolites (cotinine, *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide) and three minor tobacco alkaloids (anatabine, anabasine and nornicotine) (Fig. 1) was investigated in over 2000 urine collected during regular doping protocols, using a dilute-and-shoot sample preparation followed by ultra-high pressure liquid chromatography-triple quadrupole mass spectrometry (UHPLC–TQ-MS/MS) in hydrophilic interaction chromatography (HILIC) mode.

2. Experimental

2.1. Reagents and chemicals

(*S*)-Nicotine (≥99%) and (*S*)-cotinine (98%) were purchased from Sigma–Aldrich (Buchs, Switzerland). *trans*-3-Hydroxycotinine (99.9%), (*R*/*S*)-nicotine-*N*′-oxide (98%), (*S*)-cotinine-*N*-oxide (98%), (*R*/*S*)-anatabine (98%), (*R*/*S*)-anabasine (98%), (*S*)-nornicotine (98%) and (*R*/*S*)-*d*4-anatabine (98%) were obtained from Toronto Research Chemicals (Toronto, Canada). (*S*)-*d*4-Nicotine (98.8%), (*R*/*S*)-*d*3-cotinine (99%) and *d*3-*trans*-3-hydroxycotinine (98%) were supplied by LGC Promochem (Molsheim, France). Acetonitrile (ACN, ULC/MS quality grade, ≥99.97%) was purchased from Biosolve B.V. (Chemie Brunschwig, Basel, Switzerland) and formic acid (puriss. p.a., for HPLC, ~98%) from Fluka (Buchs, Switzerland). Ammonium formate and disodium hydrogen phosphate were supplied by Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a Quantum™ EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA).

2.2. Dilute-and-shoot sample treatment

An aliquot of urine (20 μL) was loaded by a Freedom EVO® 150 pipetting robot (Tecan Systems, Mannedorf, Switzerland) on a 96-well plate, spiked with 20 μL of deuterated internal standard (I.S.) solution (*d*4-nicotine and *d*4-anatabine at 250 ng/mL, *d*3-cotinine and *d*3-*trans*-3-hydroxycotinine at 50 ng/mL in ACN) and diluted with 760 μL ACN prior to vortex mixing, corresponding to a 40-fold dilution. After centrifugation for 5 min at 2500 rpm, the supernatant was transferred to another 96-well plate with the pipetting robot and followed by UHPLC–MS/MS injection.

When concentration of a target analyte was determined as superior to the upper limit of quantification (ULOQ), a second aliquot of urine was prepared likewise but spiked with 40 μL of I.S. solution and diluted with 1540 μL of ACN, resulting in an 80-fold dilution. Eventually, measured concentration was multiplied by 2 after UHPLC–MS/MS analysis.

2.3. UHPLC conditions

Separation was carried out on an Acquity UPLC System (Waters, Milford, USA) with a Waters Acquity UPLC BEH HILIC column (2.1 mm × 50 mm, 1.7 μm) preceded by a Waters Acquity UPLC BEH HILIC VanGuard pre-column (2.1 mm × 5 mm, 1.7 μm). Column and autosampler tray temperatures were set at 30 °C and 10 °C, respectively. Mobile phase consisted of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 800 μL/min. Initial mobile phase was 99% A held for 0.7 min, decreased linearly to 75% over 1.9 min in a first step and then to 40% over 0.35 min. The column was washed during 0.25 min and mobile phase increased back to 99% to re-equilibrate the system for 1.8 min. Injection volume was fixed at 2 μL in full loop mode.

2.4. Triple Quadrupole-MS parameters

Analyses were performed using a Waters Xevo™ TQ-S triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode. MS operating conditions were set as follows: desolvation gas flow set at 600 L/h at a temperature of 550 °C, capillary voltage at 3.0 kV in positive mode, cone voltage and collision energies optimized for each

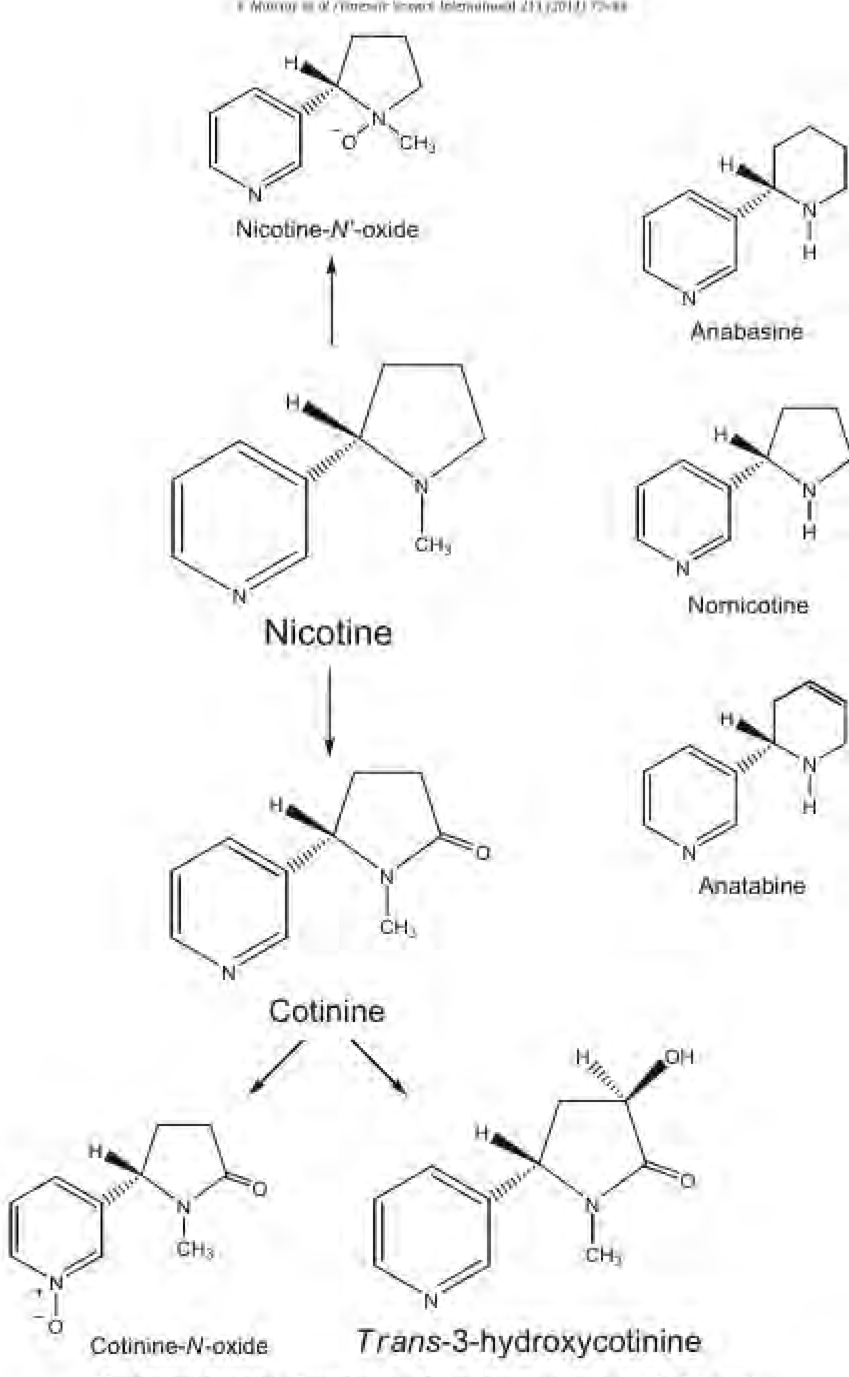
Nicotine-N'-oxide
Anabasine
Nicotine
Nornicotine
Anatabine
Cotinine
Cotinine-N-oxide
Trans-3-hydroxycotinine

Table 1
MRM parameters and retention times of the analytes.

Analyte	MRM transition (m/z)[a]	Collision energy (eV)	Cone voltage (V)	t_R (min)
Nicotine	163 → **132**, 117, 84	15	18	2.04
*d*4-Nicotine	167 → 136, **134**, 121	20	20	2.05
Cotinine	177 → **146**, 98, 80	16	15	1.02
*d*3-Cotinine	180 → 146, **101**, 81	20	20	1.04
trans-3-Hydroxycotinine	193 → **134**, 96, 80	20	16	0.97
*d*3-*trans*-3-Hydroxycotinine	196 → **134**, 99, 80	20	20	0.99
Nicotine-*N*′-oxide	179 → **132**, 130, 117	14	30	2.75
Cotinine-*N*-oxide	193 → 134, **96**, 79	18	14	1.90
Anabasine	163 → 146, 130, **117**	20	40	2.23
Anatabine	161 → 144, 117, **107**	12	26	2.13
*d*4-Anatabine	167 → 148, 132, **111**	20	20	2.13
Nornicotine	149 → 132, **117**, 106	20	34	2.27

[a] Quantification ion transitions are in bold.

compound (Table 1). The source temperature was 150 °C, the cone gas flow was set to 150 L/h and the collision gas flow was 0.15 mL/min.

2.5. Identification criteria

Identification criteria were defined in compliance with mandatory recommendations formulated in the WADA Technical Document addressing qualitative assays [19]. The retention time (t_R) tolerance window corresponding to the analyte and the quality control (QC) of the same batch must be within the range of ±2%. Also, three diagnostic ions are required for identification purpose and may include the precursor ion, with an intensity ≥5% of the most intense diagnostic ion of the MS/MS spectrum. Eventually, a Signal-to-Noise ratio ≥3 must be observed, with a relative intensity of any of the ions not differing by more than 10% (absolute) or 25% (relative) from the QC urine.

2.6. Method validation

2.6.1. Calibration curves

A method validation approach was adopted considering guidelines on bioanalytical method validation from the US Food and Drug Administration (FDA) and the recommendations of the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 [20,21].

A pool of six urine samples from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days was prepared to obtain negative urine (U_{neg}) for the validation process.

Calibration was established over the 10–10,000 ng/mL range for nicotine, cotinine and *trans*-3-hydroxycotinine and 10–5000 ng/mL for nicotine-*N*′-oxide, cotinine-*N*-oxide, anabasine, anatabine and nornicotine. A set of three validation series was achieved, with calibration standards at six concentration levels ($k = 6$), with an additional point at the highest calibration level after an 80-fold dilution, and validator standards (QC) at four concentration levels ($k = 4$), each being prepared in triplicate ($n = 3$). Calibration curves were built from the peak area ratio of nicotine to *d*4-nicotine, cotinine to *d*3-cotinine, *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide to *d*3-*trans*-3-hydroxycotinine, nornicotine, anatabine and anabasine to *d*4-anabasine. Trueness was expressed as the relative bias and defined as a ratio between the theoretical and the average measured concentration. Repeatability was defined as the relative standard deviation (RSD) of the ratio of the intra-day standard deviation and the theoretical value at each concentration [22]. Intermediate precision was expressed as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration. Accuracy profiles were built for each compound of interest, combining accuracy and intermediate fidelity variance in the dosing range [23,24].

The lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) were determined as the lowest and the highest concentrations of QC samples with an acceptable trueness, repeatability and intermediate precision. Quantitative analysis of target compounds in real urine specimens was performed using a three-points calibration curve determined and fitted by a linear least-squares regression of the peak area ratio of the analyte and the I.S. versus concentrations. The limit of detection (LOD) was defined as the concentration that produced a Signal-to-Noise ratio ≥3.

2.6.2. Selectivity

Selectivity assessment of interfering endogenous matrix compounds within selected tolerance windows was carried out by analyzing urine specimens certified as negative (≤LOD) regarding nicotine and metabolites. For this purpose, a triplicate sample treatment of urine samples from 6 nicotine-abstinent subjects who reported no exposure to environmental smoke within the last 5 days was conducted.

Accordingly, influence of exogenous xenobiotics was considered when a substance appearing on the 2010 Prohibited List and Monitoring Program was highlighted by routine sample screening procedures.

2.6.3. Matrix effects

Evaluation of matrix effects on the ionization response and extraction efficiency was achieved in regards to recommendations published elsewhere [25]. A neat solution was fortified at low, medium and high concentration in the initial mobile phase ACN: ammonium formate 10 mM (pH 3.0) buffer (99:1) (a), along with a pool of six urine samples from nicotine-abstinent subjects fortified in triplicate prior to sample treatment (b). By comparing the absolute peak areas of aqueous and urine solutions, matrix effect can be assessed, as reported below (Eq. (1)).

$$\%\ \text{Matrix effect (ME)} = \frac{b}{a} \quad (1)$$

Adopting a dilute-and-shoot approach, no extraction procedure was required, resulting in interchangeable process efficiency (PE) and matrix effect (ME). Indeed, with this particular sample treatment, ME may only be attributed to ionization of the analytes.

2.6.4. Carry-over

Injection of a blank urine sample after analysis of the highest calibrator allowed assessing presence of target compounds due to carry-over effects. This experiment was conducted in triplicate

2.6.5. Stability

The effect of storage conditions was evaluated with stability assays designed to mimic the routine analytical throughput of samples. Analyte stability was studied by monitoring the influence of 3 successive freeze and thaw cycles of QC urine samples ($n = 3$) at low and high concentrations within a week. Since real urine samples were stored at −20 °C in a dark room after collection, QCs were handled likewise and defrosted at ambient temperature 3 consecutive times within a week prior to LC–MS/MS analysis. The initial integrated peak area was defined as 100%. Similarly, short-term temperature stability was assessed for QCs laying on the bench top at room temperature (21 °C) for 24 h and in the autosampler at 10 °C for 24 h.

3. Results and discussion

3.1. Method development

3.1.1. UHPLC–MS/MS analysis

Compounds of interest, including nicotine and phase 1 metabolites along with minor tobacco alkaloids, were selected to highlight recent consumption of tobacco but also to gather comprehensive information on metabolism patterns to help distinguish between smoke and smokeless consumption in a future retrospective study. While phase II glucuronide conjugates of some metabolites may be excreted predominantly in urine, these compounds were not investigated. Indeed, the primary focus was on concentrations of nicotine with potential benefits on sport performance and relevant sensitivity was ensured for all phase 1 metabolites. Noteworthy, analysis of phase II glucuronide conjugates would require an additional hydrolysis step, which would not have completely allowed a sample treatment as straightforward as the dilute-and-shoot approach. Also, while an abundant literature on LC–MS/MS methods for the quantification of nicotine and selected metabolites in biological fluids has been published, only a handful of publications propose a simple and time-efficient sample treatment followed by fast analysis of a broad range of nicotine metabolites [26–28].

Chromatographic and detection conditions were optimized to satisfy identification criteria while allowing a high analytical throughput as favored in dope testing. Accordingly, separation of nicotine, related metabolites and minor alkaloids in urine was found successful with HILIC, using a 50 mm column length and a gradient of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 800 µL/min (Fig. 2). Indeed, combining a high flow rate and a short HILIC column with adequate retention properties towards polar molecules and excellent peak shape offered a valuable association of short analysis time while maintaining good resolution. Interestingly, ionization of target compounds was optimized by use of a mobile phase highly enriched in polar organic solvent, which led to an increase of sensitivity [29]. Also, coupling a TQ-MS analyzer to a UHPLC system brought a significant contribution to the enhancement of Signal-to-Noise ratio. Indeed, multiple reaction monitoring (MRM) acquisition mode provided an efficient isolation of characteristic fragment ions for each molecule; resulting in reduced endogenous matrix interferences. Therefore, analytical conditions ensured the suitability of a dilute-and-shoot sample treatment for urinary concentrations of nicotine, metabolites and minor alkaloids.

Likewise, triple quadrupole MS/MS parameters were optimized by direct infusion of individual standard solutions. In consequence, compound specific normalized collision energy, cone voltage and dwell time were automatically tuned, producing a high sensitivity fragmentation pattern with a precursor ion response <10% in abundance (Table 1).

Eventually, repeatability of the retention times (t_R) was assessed by calculating the relative standard deviation (RSD) of each target compound over the set of three validation series, accounting for 21 urine specimens (Table 1). Actually, fluctuations in the chromatographic conditions, including freshly prepared mobile phases, may influence the variability of t_R. Nevertheless, stability of t_R was found satisfactory, as illustrated by a RSD ranging from 0.3 to 1.7% depending on the analyte.

3.1.2. Dilute-and-shoot sample treatment

Dilute-and-shoot provided an interesting approach to the problem of finding a cost and time efficient sample treatment procedure when dealing with the consistent flow of urine specimens and restricted reporting time particular to dope testing [30–32]. Indeed, this simple method involved only limited manipulation of sample, with feasibility for automation which proved efficient for performing large batches of analyses. Coupled with short chromatographic run times allowed by UHPLC, a significant workflow of analysis was achieved. Noteworthy, this method is accepted by WADA, as mentioned in the International Standards for Laboratories (ISL) [33].

Since dilute-and-shoot sample treatment is non-selective, as opposed to solid-phase (SPE) or liquid–liquid (LLE) extraction, a variety of matrix compounds may compete with target analytes for ionization and account for substantial ME. Optimization of the dilution factor was carried out to reduce this phenomenon while maintaining sufficient sensitivity. In consequence, a 40-fold dilution appeared to provide an adequate compromise between these two parameters within the dynamic range of the mass analyzer. Also, dilution in ACN allowed improving urine clean-up due to protein precipitation and subsequent centrifugation, proving efficient to diminish potential ME. Therefore, chromatographic column lifetime could be extended and maintenance rate decreased compared to reversed phase chromatography where water is generally used for dilute-and-shoot sample treatment.

Eventually, dilute-and-shoot sample treatment provided a green alternative to SPE and LLE by requiring a low volume of organic solvent and avoiding addition of chemicals. Regarding the need of a mobile phase highly enriched in ACN for HILIC mode, this parameter was particularly valuable.

3.2. Assay validation

3.2.1. Calibration curves

Considering the pharmacological properties of nicotine and a doping perspective, concentration ranges were determined to comprehend urinary levels relevant for assessing recent consumption [34,35]. Accordingly, great efforts to develop an extensive clean-up procedure of urine samples could be avoided as quantification down to trace levels corresponding to environmental tobacco exposure or end of excretion phase after active consumption was unnecessary. Noteworthy, a fit-for-purpose approach was adopted to determine the dilution factor, which could be lowered to achieve higher sensitivity and address the later problem.

Calibration curves were built with calibration standards at six concentration levels (10-10,000 ng/mL for nicotine, cotinine and trans-3-hydroxycotinine and 10–5,000 ng/mL for nicotine-N0-oxide, cotinine-N-oxide, anabasine, anatabine and nornicotine), while evaluating different curves fitting. Referring to the accuracy profiles established over the dosage range, linear least-squares regression with $1/x^2$ weighting was chosen for quantification purpose, with coefficients of determination (R^2) greater than 0.995 (Fig. 3). Repeatability and intermediate precision met the guidelines for bioanalytical method validation over the whole assay range, with RSD values lower than 15% (Table 2). Also, trueness was found acceptable for all compounds of interest, with measured

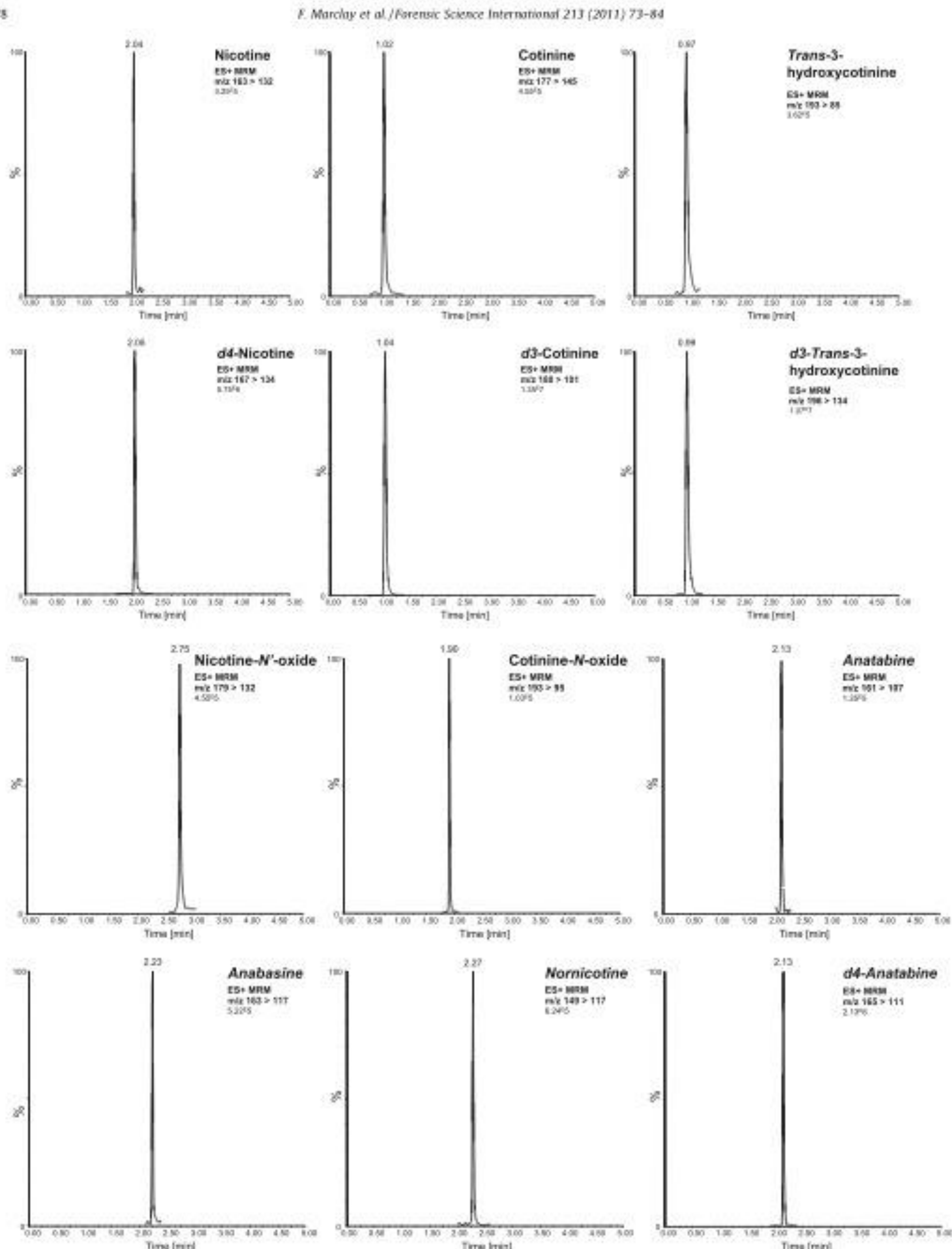

Fig. 2. UHPLC–MS/MS chromatogram of a urine specimen containing nicotine, nicotine metabolites and tobacco-related alkaloids at a concentration of 50 ng/mL, with I.S. spiked at 250 ng/mL for *d*4-nicotine and *d*4-anatabine and 50 ng/mL for *d*3-cotinine and *d*3-trans-3-hydroxycotinine.

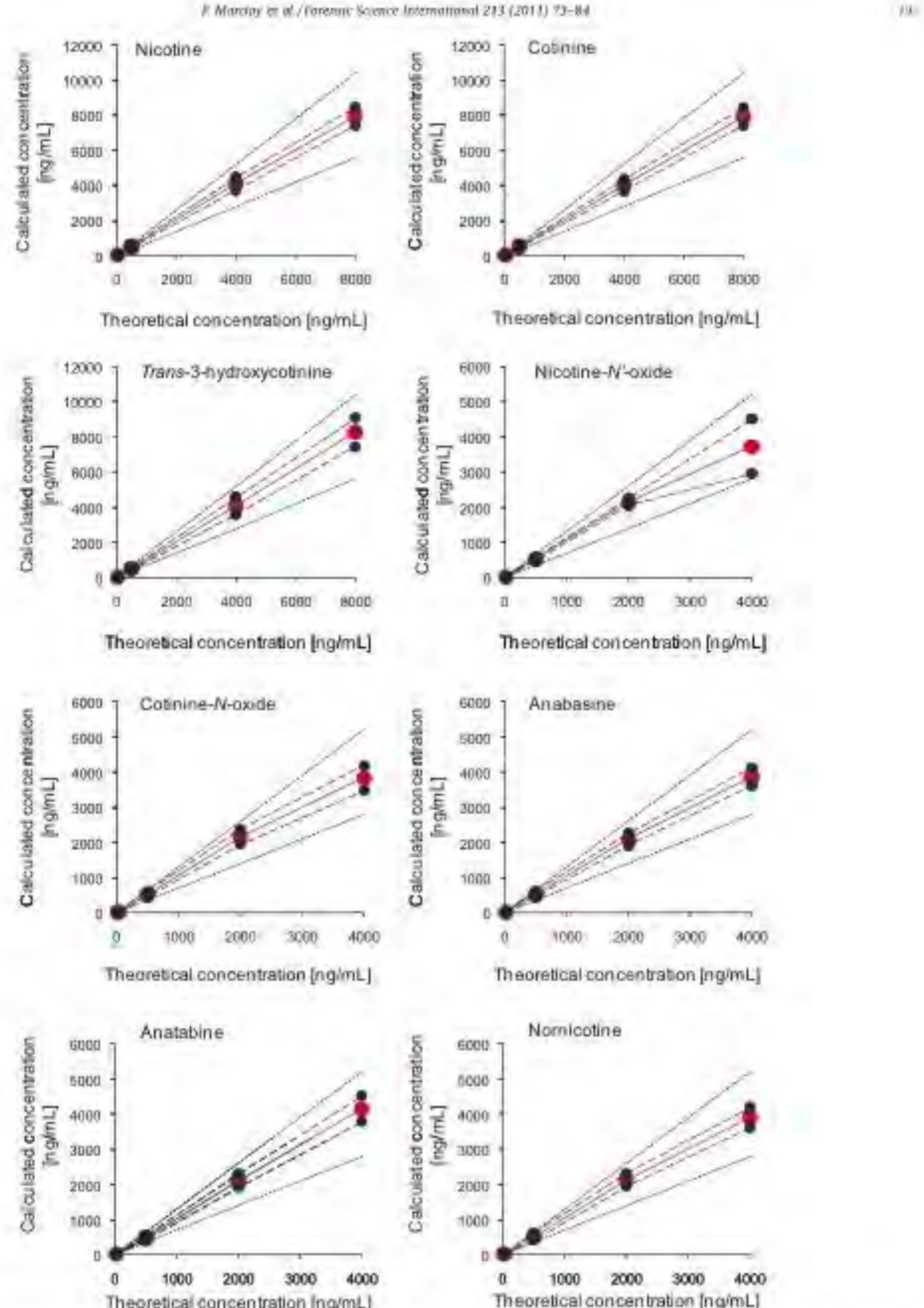

Fig. 3. Absolute accuracy profiles for nicotine, nicotine metabolites and tobacco-related alkaloids. The solid line indicates the trueness and the dashed lines represent the accuracy calculated as confidence interval [24]. The dotted lines depict the acceptance limits of ±30%.

Table 2
Assay validation parameters for nicotine and metabolites (n = 3).

Analyte	Concentration (ng/mL)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
Nicotine	20	103.7	6.2	6.7
	500	101.1	6.3	8.3
	4000	102.5	3.8	5.4
	8000	99.3	3.1	3.8
Cotinine	20	101.9	5.5	5.9
	500	101.6	7.5	6.8
	4000	100.4	2.9	4.7
	8000	96.1	3.0	5.5
trans-3-Hydroxycotinine	20	95.9	5.0	5.0
	500	100.8	7.0	9.3
	4000	102.1	4.8	7.0
	8000	103.0	3.9	5.5
Nicotine-N′-oxide	20	77.7	3.1	8.0
	500	103.6	6.2	6.2
	2000	107.5	2.4	2.4
	4000	93.2	3.2	3.2
Cotinine-N-oxide	20	99.9	8.5	6.8
	500	104.5	6.8	9.1
	2000	108.1	2.3	6.3
	4000	95.8	3.0	5.1
Anabasine	20	105.5	4.0	4.1
	500	103.8	7.4	9.8
	2000	104.3	4.8	5.3
	4000	96.8	2.9	3.6
Anatabine	20	104.1	8.1	9.9
	500	97.0	7.5	8.2
	2000	105.7	4.3	5.5
	4000	104.0	3.7	5.2
Nornicotine	20	97.4	5.2	8.4
	500	104.2	7.6	10.2
	2000	106.7	4.3	5.2
	4000	97.8	3.1	4.1

concentrations within ±15% of every theoretical concentration. Noteworthy, an 80-fold dilution proved valid for concentrations exceeding the ULOQ.

Therefore, direct quantification of target analytes in urine with this UHPLC–MS/MS method was suitable over the assay range.

Eventually, a LOD standing around 1 ng/mL for all compounds could be estimated.

3.2.2. Selectivity

Selectivity evaluation towards interfering endogenous matrix compounds was conducted on 6 different urine specimens from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days. According to triplicate sample treatment followed by UHPLC–MS/MS analysis, no interfering endogenous molecules were highlighted within selected transition windows as ion identification criteria were not met [19]. Indeed, retention times, ion transitions and ion ratios parameters observed for matrix components significantly differed from each compound of interest.

Likewise, potential influence of exogenous xenobiotics was assessed relying on routine screening analysis of every urine specimen, searching for over 200 substances appearing on the 2010 Prohibited List and Monitoring Program, followed by sample treatment and UHPLC–MS/MS analysis. Noteworthy, presence of stimulants commonly found in sport and society was investigated, including caffeine and pseudoephedrine. Accordingly, no exogenous xenobiotic highlighted by the screening procedure interfered with target analytes within selected transition windows, referring to the criteria mentioned earlier.

3.2.3. Matrix effect

Comparison of signals detected in urine and in the neat solution highlighted concentration-dependant ion enhancement or suppression effects depending on the compound of interest. Indeed, evaluation of ME indicated ion suppression occurring at low level in the 70–98% range due to endogenous matrix compounds competing with target analytes towards ionization. Despite this phenomenon could be expected from a dilute-and-shoot approach, the sample treatment method proved fit-for-purpose, as illustrated by the good repeatability of these measurements (RSD < 15%) and the sufficient sensitivity to quantify all substances at the LLOQ. Likewise, all compounds showed substantial ion enhancement at medium and high concentrations, ranging from 104 to 124% and 107 to 125%, respectively, with evidence of good repeatability (RSD < 15%). Noteworthy, ME was efficiently corrected by the deuterated standards at all concentration levels. Therefore, influence of ME on the quantitative analyses was found negligible.

3.2.4. Carry-over

Carry-over was assessed by injecting a blank urine sample following the analysis of the highest calibrator for each compound of interest. This experiment was repeated three successive times. Presence of target analytes was not detected, confirming potential carry-over effects as a negligible phenomenon.

3.2.5. Stability

Analyte stability was evaluated after investigation on the influence of the routine analytical throughput on compounds of interest in QC samples ($n = 3$) at low and high concentrations. Since stability experiments were designed to mimic actual storage and analysis conditions, QCs were preserved at −20 °C in a complete dark environment followed by a series of 3 successive freeze and thaw cycles within a week. Likewise, QC samples were left on the bench top at room temperature (21 °C) for 24 h and in the autosampler at 10 °C for 24 h to assess short-term temperature stability.

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According to minimal variations observed in the peak areas for analyte stability and short-term temperature stability assessments (RSD < 15%), storage conditions adopted during this study were adequate to ensure a high stability of all compounds of interest.

3.3. *Application to doping control samples in 2010–2011*

Over a one-year period of time covering 2010 and 2011, every single urine specimen from regular doping controls of 43 different disciplines analyzed by the Swiss Laboratory for Doping Analyses (LAD) was included in this monitoring ($n = 2185$). Referring to the 2009 International Standards for Laboratories (ISL), article 19 of the World Anti-Doping Code and articles 24–27 of the UNESCO Convention against doping in sport, complete removal of identification means and a minimum storage period of three months were ensured prior to initiation of this research [33,36,37]. Also, approval was received from Antidoping Switzerland (ADS) to conduct this study.

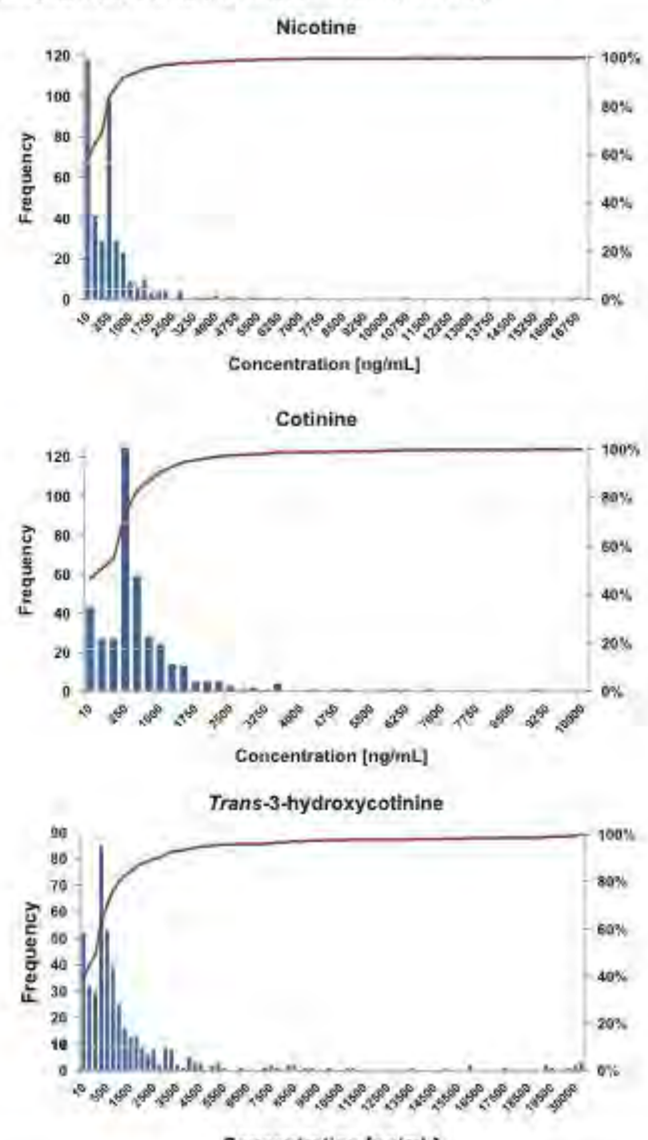

Fig. 4. Concentrations distribution for nicotine, nicotine metabolites and tobacco-related alkaloids. The solid line indicates the cumulative percentage.

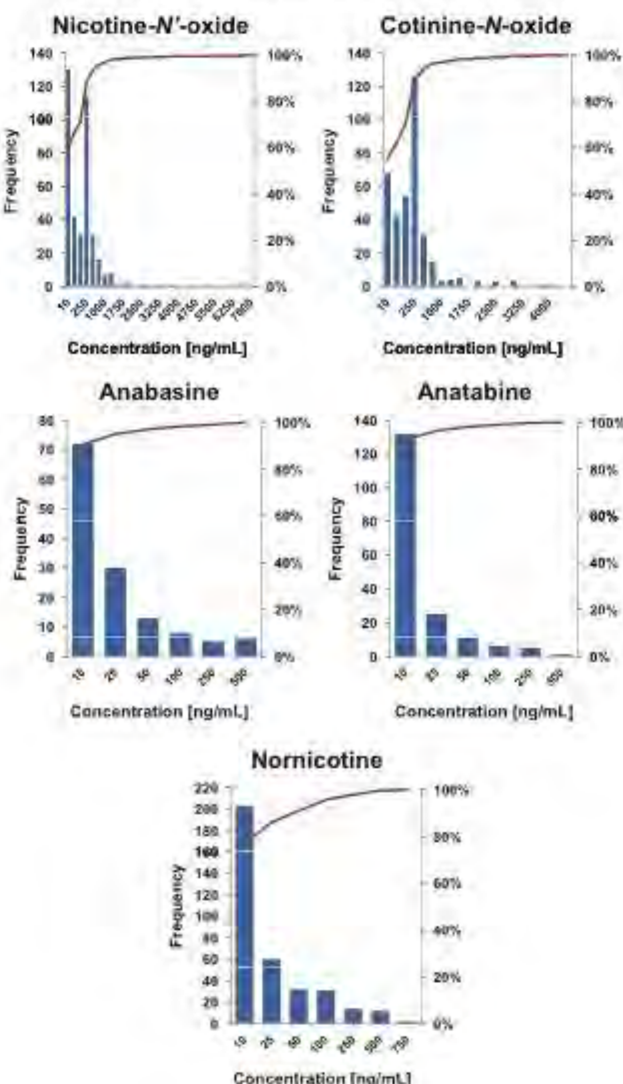

Fig. 4. (Continued).

Due to the linear response observed after $1/x^2$ weighting in the assay validation, quantification was performed using calibration standards reduced to LLOQ, medium and ULOQ concentration levels ($k = 3$) and QCs to low and high concentration levels ($k = 2$), prepared each in triplicate. Also, a qualitative designation was assigned to compounds of interest detected in the LOD to LLOQ concentration range, namely traces.

According to the quantitative assays, concentrations distribution of major nicotine metabolites, minor nicotine metabolites and tobacco alkaloids were found to range from LLOQ to 32,223, 6670 and 538 ng/mL, respectively (Fig. 4). Traces of major nicotine metabolites, minor nicotine metabolites and minor tobacco-related alkaloids accounted for 17.9–20.5%, 16.5–17.9% and 6.1–16.2% of urine specimens, respectively (Table 3). Actually, at least one of the five different metabolites was detected in 23.0% of samples. Thus, exposure to nicotine, either due to active consumption or environmental tobacco smoke, concerned approximately twenty-three athletes out of a hundred. Considering a worldwide smoking prevalence of approximately 25%, as reported by the World Health Organization (WHO), and huge progress towards implementation of a smoke-free environment, such findings support the potential use of nicotine in sport with a

Table 3
Prevalence of doping urine samples exposed to nicotine, nicotine metabolites and tobacco alkaloids depending on the concentration range ($n = 2185$).

Analyte	Concentration range		
	LOD ≤ x ≤ LLOQ	LLOQ ≤ x ≤ ULOQ	Active consumption
Nicotine	17.9%	12.5%	9.3%
Cotinine	18.0%	16.1%	13.6%
trans-3-hydroxycotinine	20.5%	18.1%	15.3%
Nicotine-N′-oxide	17.9%	13.4%	10.0%
Cotinine-N-oxide	15.5%	11.9%	11.4%
Anabasine	6.1%	2.6%	1.5%
Anatabine	8.2%	2.2%	1.1%
Nornicotine	16.2%	6.9%	4.2%
Cumulative exposure	23.0%	18.3%	15.3%

Table 4
Cumulative exposure to nicotine, nicotine metabolites and tobacco alkaloids in doping urine samples of selected sports.

Sport	n of samples	Cumulative exposure		
		LOD ≤ x ≤ LLOQ	LLOQ ≤ x ≤ ULOQ	Active consumption
American football	19	55.6%	55.6%	55.6%
Basketball	24	33.3%	25.0%	25.0%
Biathlon	38	23.7%	21.1%	18.4%
Bobsleigh	78	38.5%	33.3%	30.8%
Football	205	28.8%	23.9%	19.0%
Gymnastics	48	37.5%	31.3%	29.2%
Ice hockey	108	45.4%	37.0%	32.4%
Rugby	25	28.0%	28.0%	28.0%
Skating	41	29.3%	19.5%	19.5%
Skiing	143	35.7%	28.7%	25.9%
Volleyball	46	26.1%	21.7%	19.6%
Wrestling	31	45.2%	35.5%	32.3%

specific purpose [38,39]. When focusing on sports with a significant number of samples submitted to dope testing, cumulative exposure to nicotine metabolites and tobacco alkaloids was found to range between 26.1 and 55.6% of urine specimens for ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics (Table 4).

Likewise, above-LLOQ levels of major nicotine metabolites, minor nicotine metabolites and minor tobacco-related alkaloids were quantified in 12.5–18.1%, 11.9–13.4% and 2.2–6.9% of urine specimens, respectively. At least one of the five different metabolites was measured in this concentration range in 18.3% of samples. As such, approximately eighteen athletes out of a hundred were exposed to smoke or smokeless tobacco or environmental tobacco exposure (ETS) within the last 3 days before doping control [16]. Regarding the specific disciplines mentioned previously, recent cumulative exposure concerned 19.5–55.6% of urine specimens.

Eventually, measuring prevalence of nicotine consumption prior to or/and during sport practice was carried out by hypothesizing conservative concentration limits for active exposure (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-N′-oxide, cotinine-N-oxide, anabasine, anatabine and nornicotine), as depicted elsewhere [16,40,45]. Likewise, since extensive exposure to second-hand smoke shortly before sport practice was extremely unlikely, contribution of ETS to such levels of concentration could be excluded. Thus, referring to those concentration ranges and considering major nicotine metabolites, minor nicotine metabolites and minor tobacco-related alkaloids, consumption of nicotine associated to sport training or competition was highlighted in 9.3–15.3%, 10.0–11.4% and 1.1–4.2% of urine specimens, respectively. Noteworthy, at least one of these compounds of interest was present in such concentrations in 15.3% of samples. Accordingly, about fifteen athletes out of a hundred were considered as active nicotine consumers in this monitoring study, regardless of the sport discipline. However, while such statistics may appear lower than smoking prevalence in society, putting emphasis on ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics indicated a consumption prevalence comparable, if not far superior, to regular recreational use. Indeed, active exposure has been highlighted for 19.0–55.6% of urine specimens, depending on the sport. Therefore, such numbers denote more extensive and alarming exposure levels, which may result from a social trend within a particular community of athletes combined with pharmacological properties of nicotine providing particularly interesting performance benefits in specific disciplines. Likewise, with respect to the detrimental effects of smoking on the respiratory system, these statistics bring a very significant support to the hypothesis of smokeless tobacco use as a performance enhancer, in particular for the sports highlighted in this survey [41–44].

At present, a more definite number for prevalence of smokeless tobacco use could not be determined as there is no reported clinical study proposing a metabolic-based approach to distinguish between different forms of nicotine consumption. Nevertheless, a retrospective study after future development of statistical tools appears feasible as a large number of compounds related to the metabolism of nicotine or tobacco alkaloids have been quantified.

Therefore, considering a public health perspective, a preventive rather than repressive attitude in fight against doping may be an interesting and innovative approach to tackle this alarming consumption phenomenon. Indeed, inclusion of nicotine to the Prohibited List or/and Monitoring Program may not only prevent consumption of this molecule as a performance enhancer but also significantly limit initiation and development of drug addiction during sport practice and pursuit after a professional career.

4. Conclusion

As a response to smoking prohibition policies flourishing throughout the world, the tobacco industry initiated a strong

marketing process of various smokeless products, in particular snus. With progression of consumption in society, smokeless tobacco is drawing attention in the sport community due to performance enhancement pharmacological properties of nicotine free of smoke-related detrimental effects on the respiratory tract. However, despite the so-claimed reduced health risk, such products induce persistent addiction issues and may be responsible for a vast panel of diseases, including different types of cancers.

Thus, in order to measure the size of this phenomenon, a sensitive, selective and suitable UHPLC–MS/MS method operated in HILIC mode for the detection and quantification of nicotine, its four principal metabolites and related tobacco alkaloids in urine was developed and validated. A straight-forward sample treatment procedure based on a dilute-and-shoot approach was favored, providing a very significant workflow of analysis, especially as coupled with short run times allowed by UHPLC and very good chromatographic performance, peak shape and enhanced sensitivity associated with HILIC mode. With a limited reporting time and a consistent flow of samples in dope testing, such methodology proved particularly efficient.

Subsequently, prevalence of nicotine consumption amongst athletes during a one-year monitoring study between 2010 and 2011, accounting for 2185 urine specimens of 43 different sports, was investigated with this analytical procedure. Traces of major and minor nicotine metabolites as well as minor tobacco-related alkaloids were detected in approximately twenty-three urine specimens out of a hundred, with concentration levels corresponding to exposure within the last three days for about eighteen specimens out of a hundred. Eventually, prevalence of nicotine consumption, as a smoke or smokeless tobacco product, before or/and during sport practice revealed that about fifteen athletes out of a hundred were active users.

As a first approach, this number may appear lower than the worldwide smoking prevalence of around 25%. However, focusing the study on a panel of selected sports brought alarming evidence on comparable, if not far superior, nicotine consumption amongst athletes. Indeed, prevalence in ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics was found to range between 19.0 and 55.6%. Since smoking may be responsible for noticeable respiratory effects and numerous health threats detrimental to sport practice at top level, likelihood of smokeless tobacco consumption for performance enhancement is a hypothesis of very serious concern. While the frontier between recreational consumption and use for doping purpose is difficult to ascertain with social drugs, including nicotine, caffeine or tetrahydrocannabinol (THC), toxicity of tobacco products is responsible for disastrous health effects greatly amplified by persistent addiction issues.

Therefore, WADA and sport federations should evaluate the inclusion of nicotine to the Prohibited List or/and Monitoring Program in order to bring not only control on a doping agent, but also an innovative and key element to developing a more preventive approach of fight against doping. Indeed, an interesting step towards limitation and education on a global public health threat responsible for an extremely harmful burden of disease could be initiated.

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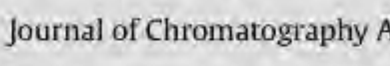

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Determination of nicotine and nicotine metabolites in urine by hydrophilic interaction chromatography–tandem mass spectrometry: Potential use of smokeless tobacco products by ice hockey players

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A B S T R A C T

Consumption of nicotine in the form of smokeless tobacco (snus, snuff, chewing tobacco) or nicotine-containing medication (gum, patch) may benefit sport practice. Indeed, use of snus seems to be a growing trend and investigating nicotine consumption amongst professional athletes is of major interest to sport authorities. Thus, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the detection and quantification of nicotine and its principal metabolites cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide in urine was developed. Sample preparation was performed by liquid–liquid extraction followed by hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS) operated in electrospray positive ionization (ESI) mode with selective reaction monitoring (SRM) data acquisition. The method was validated and calibration curves were linear over the selected concentration ranges of 10–10,000 ng/mL for nicotine, cotinine, *trans*-3-hydroxycotinine and 10–5000 ng/mL for nicotine-*N'*-oxide and cotinine-*N*-oxide, with calculated coefficients of determination (R^2) greater than 0.95. The total extraction efficiency (%) was concentration dependent and ranged between 70.4 and 100.4%. The lower limit of quantification (LLOQ) for all analytes was 10 ng/mL. Repeatability and intermediate precision were ≤9.4 and ≤9.9%, respectively. In order to measure the prevalence of nicotine exposure during the 2009 Ice Hockey World Championships, 72 samples were collected and analyzed after the minimum of 3 months storage period and complete removal of identification means as required by the 2009 International Standards for Laboratories (ISL). Nicotine and/or metabolites were detected in every urine sample, while concentration measurements indicated an exposure within the last 3 days for eight specimens out of ten. Concentrations of nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide were found to range between 11 and 19,750, 13 and 10,475, 10 and 8217, 11 and 3396, and 13 and 1640 ng/mL, respectively. When proposing conservative concentration limits for nicotine consumption prior and/or during the games (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-*N'*-oxide and cotinine-*N*-oxide), about half of the hockey players were qualified as consumers. These findings significantly support the likelihood of extensive smokeless nicotine consumption. However, since such conclusions can only be hypothesized, the potential use of smokeless tobacco as a doping agent in ice hockey requires further investigation.

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1. Introduction

Nicotine is the principal natural alkaloid present in tobacco leaves. A wide variety of consumption patterns exist, from tobacco smoking, in the form of cigarettes, cigars or pipes, to smokeless tobacco products such as snus, snuff and chewing tobacco. Nicotine replacement therapies also contain this natural compound, as marketed in transdermal patches, nasal sprays, inhalers and gums [1].

Depending on the type of product, concentrations differ to a reasonable extent. On average, a similar content of nicotine is found in cigarette and oral snuff, whereas cigar and chewing tobacco contain only about half of this concentration [1]. Accordingly, levels of nicotine intakes and metabolism pathways vary along these different trends of tobacco consumption. When smoked and inhaled, nicotine is rapidly absorbed in the lungs, reaching the brain via the bloodstream within 20 s [1]. Depending on the pH, there is little to large buccal absorption, which is directly related to the type of product [2,3]. Chewing tobacco and snus are buffered to facilitate absorption of nicotine through oral mucosa. A portion of nicotine is usually swallowed with saliva and well absorbed in the small

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Fig. 1. Simplified metabolic pathway of nicotine [1].

intestine. Concentration in the brain rises at a slower rate than with smoking and levels are declining over a longer period of time. Nicotine is also well absorbed through the skin which is the basis for transdermal delivery that occurs over a long period of time [4].

Nicotine is primarily and extensively metabolized in the liver by *C*-oxidation to cotinine [2,5]. *N*-oxidation also converts nicotine into nicotine-*N'*-oxide and other minor metabolites. Cotinine is further hydroxylated to *trans*-3-hydroxycotinine and also converted to cotinine-*N*-oxide and other minor metabolites by *N*-oxidation (Fig. 1). Simultaneous determination of free urinary nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide account for 8–10, 10–15, 33–40, 4–7 and 2–5% of the total nicotine dose, respectively [5,6].

Due to the relatively short half-life of nicotine in urine (about 2 h), investigating nicotine metabolites which exhibit a longer half-life is a prerequisite to provide relevant information on tobacco consumption [1]. Therefore, an abundant literature on gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods has been published for the determination and quantification of nicotine and selected metabolites in biological fluids, including blood or plasma, urine and saliva [6–14].

LC–MS/MS provides a sensitive and selective approach for comprehensive measurement of free nicotine and its metabolites. However, only very few of these publications bring up a simultaneous, yet steps limited, sample preparation method for the analysis of nicotine and metabolites, in particular nicotine-*N'*-oxide and cotinine-*N*-oxide [6,9,10,12].

Nicotine can act both as a stimulant and a relaxant drug, with predominant effects being an increase in pulse rate and blood pressure, as well as an increase of blood sugar release and the release of epinephrine [4,15]. Positive reinforcing effects also include relaxation, reduced stress, enhanced vigilance, improved cognitive function, mood modulation and lower body weight [3,16].

Thus, when considering nicotine from a doping perspective, consumption in the form of smokeless nicotine products may clearly enhance the performances of sport athletes in various ways as it provides all the effects described above, without the direct health issues usually associated to smoke [17]. Indeed, use of snus, snuff or chewing tobacco has been reported as a growing trend, in particular amongst winter sports such as ice hockey and skiing, but also in other popular sports such as soccer, baseball or basketball and even in fencing or shooting [18–20]. Nevertheless, only old and vague estimates of these consumption patterns have been reported, leading to an extensive underestimate of this potential issue. Nicotine did not appear in the 2009 World Anti-Doping Agency (WADA) Prohibited List or in the 2009 Monitoring Program, a situation which remains unchanged at the present time [21,22]. Thus, investigating nicotine consumption trends amongst professional athletes and developing means to distinguish between consumption of smoke or smokeless nicotine products is of major concern to sport authorities.

Therefore, the project presented in this paper describes an analytical method for the simultaneous determination and quantification of nicotine and its four main metabolites in urine, using liquid–liquid extraction (LLE) followed by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) in Hydrophilic Interaction Chromatography (HILIC) mode. Apart from a recent publication on nicotine, cotinine and *trans*-3-hydroxycotinine analysis [23], HILIC columns have never been used for such purpose in real biological samples, in particular when including nicotine-*N'*-oxide and cotinine-*N*-oxide. However, this methodology is primarily dedicated to the analysis of polar compounds such as molecules and related metabolites excreted in urine [24,25]. Owing to the nature of screening procedures for doping agents, a rapid and simple extraction procedure is favoured for comprehensive nicotine consumption study.

This analytical approach has been further applied to the urine samples collected during the 2009 Ice Hockey World Championships held in Switzerland in order to measure the prevalence of nicotine exposure amongst athletes and help to assess the concern associated with nicotine consumption in sport.

2. Experimental

2.1. Reagents and chemicals

(*S*)-Nicotine (≥99%) and (*S*)-cotinine (98%) were purchased from Sigma-Aldrich (Buchs, Switzerland). *trans*-3-hydroxycotinine (99.9%), (*R*/*S*)-nicotine-*N'*-oxide (98%) and (*S*)-cotinine-*N*-oxide (98%) were obtained from Toronto Research Chemicals (Toronto, Canada), while (*S*)-*d*4-nicotine (98.8%), (*R*/*S*)-*d*3-cotinine (99%) and *d*3-*trans*-3-hydroxycotinine (98%) were supplied by LGC Promochem (Molsheim, France). Acetonitrile (ACN, ≥99.7%) was

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purchased from Biosolve B.V. (Chemie Brunschwig, Basel, Switzerland), propan-2-ol (≥99%) was obtained from BDH Laboratory Supplies (Poole, England), chloroform (≥99.0%) from Acros Organics (Geel, Belgium) and formic acid (~98%) from Fluka (Buchs, Switzerland). Ammonium formate and disodium hydrogen phosphate were supplied by Sigma-Aldrich (Buchs, Switzerland) and potassium dihydrogen phosphate by VWR International AG (Dietikon, Switzerland). Ultra-pure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a QuantumTM EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA).

2.2. *Sample preparation*

Urine samples clean-up is based on a method previously published and adapted to our particular needs and matrix [9]. An aliquot of urine (1 mL) was spiked with 10 μl of 10 μg/ml deuterated internal standard (I.S.) solution (*d*4-nicotine, *d*3-cotinine and *d*3-*trans*-3-hydroxycotinine) and diluted with 1 mL phosphate buffer (0.2 M, pH 7.0) prior to vortex mixing. LLE was performed with 2.5 mL chloroform:propan-2-ol (95:5, v/v) for 10 min using a rotator unit. After centrifugation for 5 min at 2500 rpm, the organic layer was evaporated to dryness under a gentle air stream at 50 °C and reconstituted in 1 mL ACN:ammonium formate (10 mM, pH 3.0) (98:2, v/v) prior to LC–MS/MS injection.

2.3. *LC conditions*

Separation was carried out on a LC–MS/MS system using a Rheos 2000 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and an HTS Pal autosampler (CTC analytics AG, Zwingen, Switzerland). Hydrophilic Interaction Chromatography was performed on a Phenomenex Luna® HILIC column (150 mm × 3.0 mm, 5 μm) (Brechbühler AG, Schlieren, Switzerland) with a guard column SecurityGuard™ HILIC (4 mm × 2.0 mm) (Brechbühler) added to the analytical column. The column temperature and the autosampler tray were set at 30 and 4 °C, respectively. Mobile phase consisted of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 0.3 mL/min, while partial loop injection volume was 10 μl with a 20 μl loop. The initial mobile phase condition was 98% A for 3 min, which was decreased linearly to 35% and held from 10 to 13 min, then increased back to 98% to re-equilibrate the column from 13.1 to 16 min.

2.4. *Linear trap quadrupole-MS parameters*

Analyses were performed using a linear ion trap mass spectrometer LTQ-MS (ThermoFinnigan, San Jose, CA, USA) equipped with an atmospheric pressure ionization (API) interface, Ion MAX™, operated in positive ESI mode. MS operating conditions were set as follows: spray voltage = 5.0 kV; heated capillary voltage and temperature of 10 V and 320 °C, respectively; isolation width of 1.5 Da; activation time = 30 ms; activation *q* of 0.250 and scan time was fixed at 30 ms. Sheath gas, auxiliary gas and sweep gas (nitrogen) were set at 20, 5 and 1.5, respectively.

2.5. *Identification criterias*

Identification criteria were defined according to the WADA Technical Document addressing this particular topic [26]. The retention time (t_R) tolerance window must be within the range of ±2% between the analyte and the QC of the same batch. Also, concerning MS/MS experiments, three diagnostic ions are required, which may include the precursor ion and with an intensity ≥5% of the most intense diagnostic ion of the MS/MS spectrum. A Signal-to-Noise ratio >3 must be considered and the relative intensity of any of the ions shall not differ by more than 10% (absolute) or 25% (relative) from that of the quality control urine.

2.6. *Method validation*

2.6.1. *Calibration curves*

Experiments were conducted following the guidelines on bioanalytical method validation from the US Food and Drug Administration (FDA) and the recommendation of the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 [27,28].

A pool of six urine samples from nicotine-abstinent individuals who had *not* been exposed to environmental smoke within the last 5 days was prepared to obtain a negative urine (U_{neg}) for the validation process.

Also, according to the pharmacological effects of nicotine and keeping in mind a doping perspective, only recent consumption of nicotine was of relevant interest. Indeed, trace levels in the 1 ng/mL scale would not provide meaningful quantitative information on the consumption behavior. Thus, in order to ensure statistical significance for further discrimination between recreational, environmental or doping use, selecting a LLOQ of 10 ng/mL through the validation procedure ensured quality quantitative results while maintaining minor bias.

Therefore, the calibration was established over the 10–10,000 ng/mL range for nicotine, cotinine and *trans*-3-hydroxycotinine and 10–5000 ng/mL range for nicotine-*N'*-oxide and cotinine-*N*-oxide. A set of three validation series was achieved, with calibration standards at six concentration levels ($k = 6$) and validator standards (QC) at four concentration levels ($k = 4$) prepared in triplicate ($n = 3$) each time. Calibration curves were built from the peak area ratio of nicotine and metabolites to *d*4-nicotine for nicotine, *d*3-cotinine for cotinine and *d*3-*trans*-3-hydroxycotinine for *trans*-3-hydroxycotinine, nicotine-*N'*-oxide and cotinine-*N*-oxide. Accuracy was expressed as the ratio between the theoretical and the average measured concentration. Repeatability was expressed as the relative standard deviation (RSD) of the ratio of the intra-day standard deviation and the theoretical value at each concentration level [29]. Intermediate precision was expressed as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration level. An accuracy profile was built for each analyte, combining accuracy and intermediate fidelity variance in the dosing range [30,31]. Data were processed and reported with Xcalibur LCQuan package software from ThermoFinnigan and calculation were performed on *Excel 2007* from Microsoft.

The lower limit of quantification (LLOQ) was determined as the lowest QC sample with an acceptable trueness, repeatability and intermediate precision fitting for purpose. Quantitative analysis of nicotine and metabolites in real urine samples was performed using a three-points calibration curve determined and fitted by a linear least-squares regression of the peak area ratios between the analyte and the IS versus concentrations. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level of a blank urine preparation.

2.6.2. *Selectivity*

Influence of endogenous matrix compounds was determined by analyzing urine samples from six individuals certified as negative (<LOD) for nicotine and metabolites. Each sample was extracted in triplicate to highlight the presence of potential interfering matrix compounds within selected tolerance windows.

Accordingly, influence of exogenous xenobiotics was determined by analyzing urine samples from over 250 individuals with

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different nicotine consumption habits who reported the use of very various substances appearing on the 2009 Prohibited List and Monitoring Program.

2.6.3. Carry-over

Carry-over was evaluated correspondingly by injecting a blank urine sample subsequently to the analysis of the highest calibrator. This experiment was conducted in triplicate.

2.6.4. Matrix effects

Matrix effects on the ionization response and extraction efficiency were further evaluated along the recommendations published elsewhere [32]. A neat solution was fortified at low, medium and high concentration in the initial mobile phase ACN:ammonium formate 10 mM (pH 3.0) buffer (98:2) (a), while a set of 6 negative urines was also fortified in duplicate prior to extraction (b) and another set of blank urine specimens was extracted and fortified only after (c). By comparing the absolute peak areas of two sets of solutions, matrix effect and extraction efficiency can be evaluated, as reported below (Eqs. (1) and (2)).

$$\text{Matrix effect (ME)} = \frac{c}{a} \quad (1)$$

$$\text{Extraction efficiency (RE)} = \frac{b}{c} \quad (2)$$

2.6.5. Stability

The effect of storage conditions was studied by performing a longitudinal stability assay. Analyte stability was evaluated by monitoring the influence of successive freeze and thaw cycles of QC urine samples at low, medium and high concentrations over a period of 6 months. As real urine samples were stored at −20 °C in a sealed box since their collection, the QCs were handled likewise and defrosted at ambient temperature twice a month for LC–MS/MS analysis. The initial integrated peak area was defined as 100%.

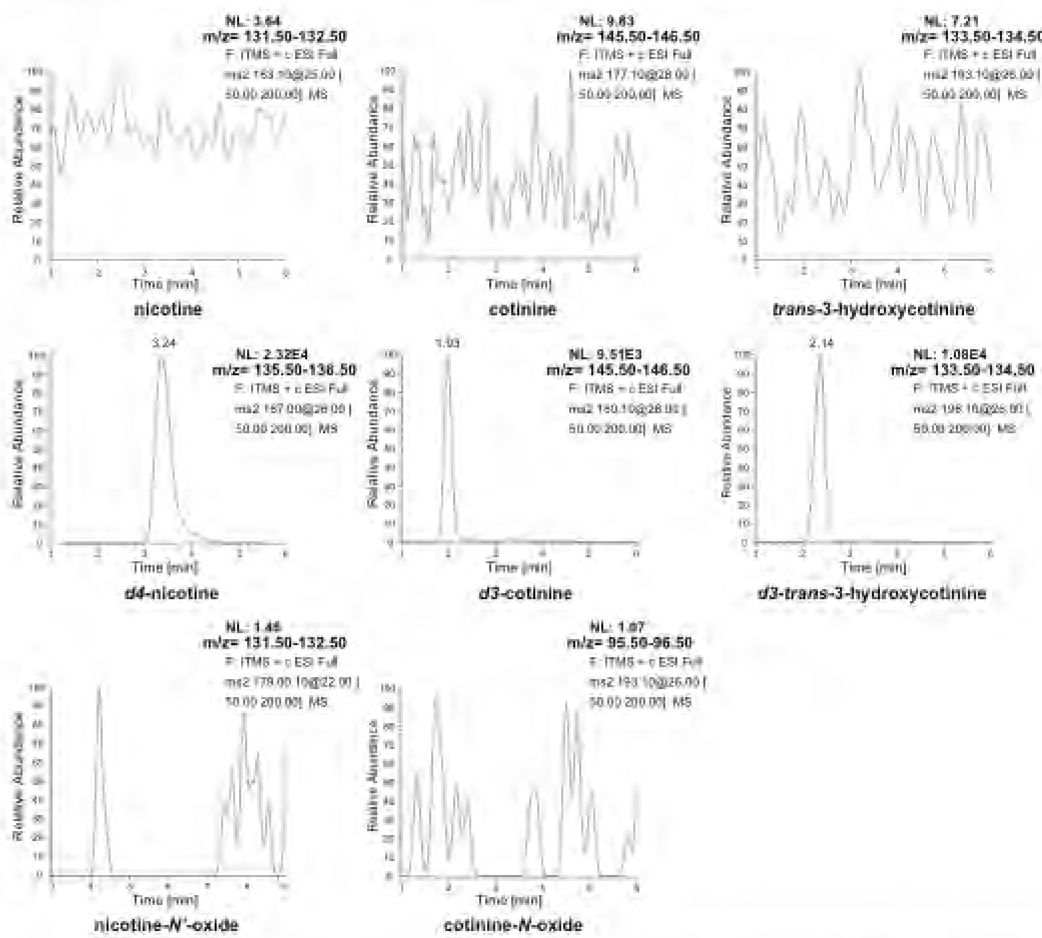

Fig. 2. LC–MS/MS chromatograms of a blank urine as opposed to a urine specimen containing nicotine, cotinine, trans-3-hydroxycotinine, nicotine-N′-oxide and cotinine-N-oxide at a concentration of 10 ng/mL, both with IS spiked at 100 ng/mL. A chromatogram of a urine sample from a hockey player containing the above mentioned metabolites at 1024, 1415, 3739, 2586 and 459 ng/mL, respectively, is also depicted. Quantification ion transitions are in bold.

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3. Results and discussion

3.1. Method development

3.1.1. LC–MS/MS analyses

A complete separation of nicotine and metabolites in urine specimens was achieved by hydrophilic interaction chromatography using a gradient of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 0.3 mL/min (Fig. 2). Indeed, HILIC mode allowed to successfully isolate each analyte by providing adequate retention of polar compounds and excellent peak shape. Sensitivity was also optimized since using a mobile phase highly enriched in polar organic solvent ensures an efficient ionization towards the molecules of interest [24]. Likewise, reduced endogenous matrix interferences resulted in very clean chromatograms and a high throughput was obtained due to the feasibility of using a higher flow rate.

Repeatability of the retention times (t_R) was evaluated by calculating mean values variability over the set of three validation series which consisted in 45 extracted samples (Table 1). The RSD obtained were found satisfactory for all the compounds of interest, ranging from 1.8 to 4.1%.

Direct infusion of individual standard solutions, with a flow rate and mobile phase composition corresponding to the elution time from the LC column, allowed optimization of tandem mass spectrometry parameters. Gas streams, spray voltage, heated capillary voltage and temperature, isolation width and compound specific normalized collision energies were manually tuned, resulting in a high sensitivity fragment spectra with a precursor ion response <10% in abundance. SRM transitions, collision energies and retention times for each analyte are provided in Table 1.

3.1.2. LLE

Sample preparation in dope testing favors time and cost efficient procedures which provide satisfactory matrix clean-up and recovery. Thus, the selective extraction protocol for urine samples used in this work was performed with a single LLE. Nicotine and metabolites were neutralized with phosphate buffer at pH 7.0, triggering the extraction with chloroform:propan-2-ol (95:5, v/v). Extraction was followed by evaporation of the organic phase and reconsti-

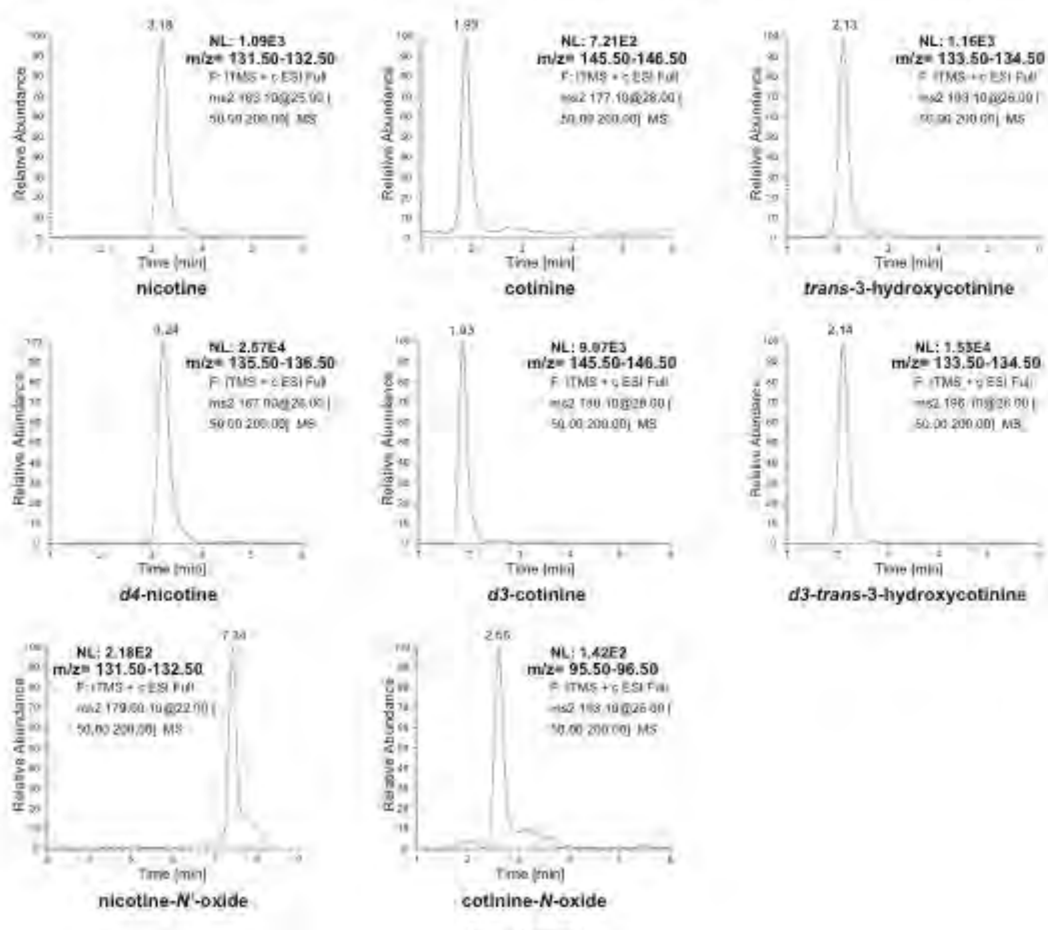

Fig. 2. (Continued)

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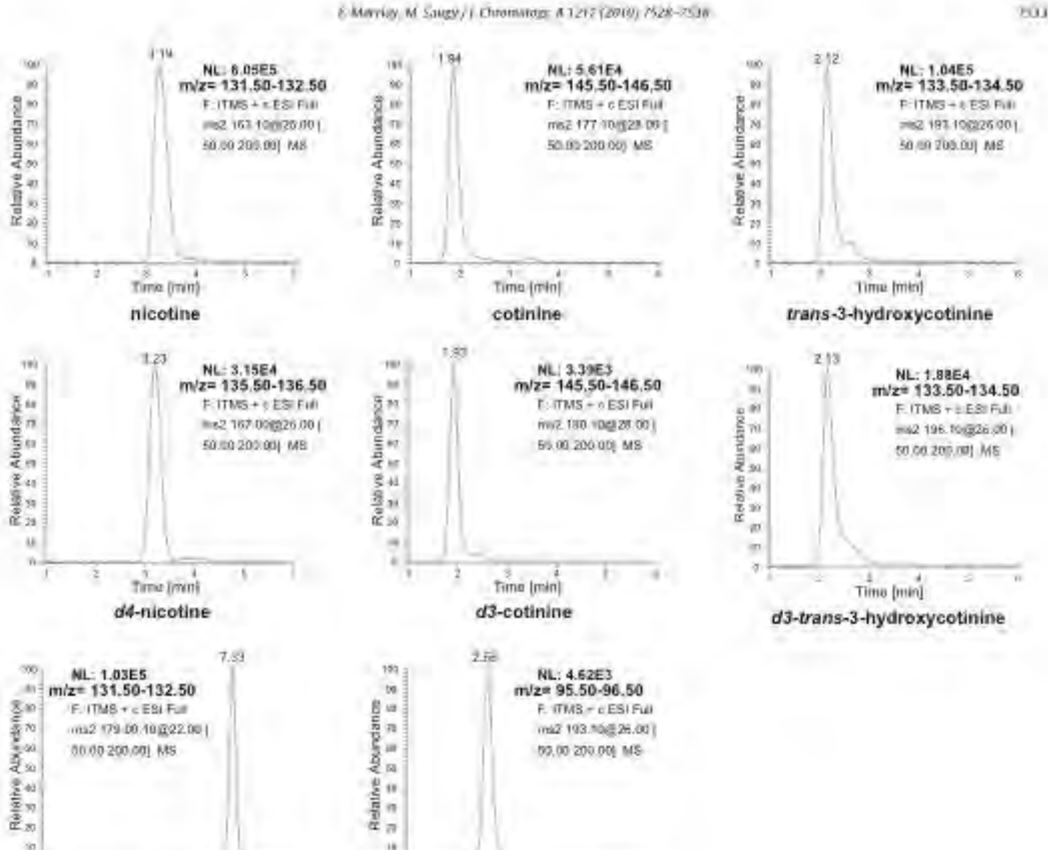

Fig. 2. (*Continued*)

Table 1
SRM parameters and retention times of the analytes.

Analyte	SRM transition (m/z)[a]	Collision energy (eV)	t_R (min)
Nicotine	163 → **132**, 120, 106	25	3.18
d4-Nicotine	167 → **136**, 124, 110	26	3.24
Cotinine	177 → **146**, 98, 80	28	1.93
d3-Cotinine	180 → **146**, 101, 81	28	1.93
trans-3-Hydroxycotinine	193 → **134**, 118, 80	26	2.13
d3-*trans*-3-Hydroxycotinine	196 → **134**, 39, 80	26	2.14
Nicotine-*N'*-oxide	179 → **132**, 130, 117	22	7.34
Cotinine-*N*-oxide	193 → 134, **96**, 98	26	2.56

[a] Quantification ion transitions are in bold.

Table 2
Recoveries of nicotine and metabolites at low and high concentrations (n = 5).

Analyte	Concentration (ng/mL)			Recovery (%)			RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Nicotine	10	5000	10,000	95.2	93.4	89.8	3	2	3
Cotinine	10	5000	10,000	99.8	97.6	95.7	0	5	1
trans-3-Hydroxycotinine	10	5000	10,000	70.4	71.8	73.1	5	3	2
Nicotine-*N'*-oxide	10	2500	5000	82.3	83.1	83.2	3	1	0
Cotinine-*N*-oxide	10	2500	5000	79.6	80.5	82.7	0	0	1

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Table 3
Assay validation parameters for nicotine and metabolites (n = 3).

Analyte	Concentration (ng/mL)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
Nicotine	10	98.4	7.9	8.1
	5000	92.5	6.5	6.6
	10,000	91.8	6.9	6.9
Cotinine	10	105.0	8.4	9.2
	5000	85.5	6.9	7.2
	10,000	109.1	2.1	6.8
trans-3-Hydroxycotinine	10	96.0	7.9	7.8
	5000	89.6	6.0	7.3
	10,000	96.1	5.8	6.2
Nicotine-N′-oxide	10	102.4	5.5	5.1
	2500	96.8	9.4	9.9
	5000	94.3	7.1	6.6
Cotinine-N-oxide	10	103.9	5.1	5.7
	2500	101.1	5.4	5.9
	5000	89.9	4.6	6.7

tution in the initial mobile phase mixture. This simple, cost and steps-limited methodology provided very clean extracts of urine samples containing nicotine and metabolites. Noteworthy, a batch of 50 items could be prepared within 1 h, allowing a significant workflow of analysis.

RE ranged from 70.4 to 100.4% depending on the analyte, with evidence of good repeatability (RSD < 15%), and showed only slight dependency on the concentration level (Table 2). Indeed, RE for *trans*-3-hydroxycotinine was below what was obtained for the other metabolites. This may result from the pK_a of *trans*-3-hydroxycotinine being much lower compared to the pH of the phosphate buffer.

3.2. Assay validation

3.2.1. Calibration curves

Concentration ranges were initially determined according to expected levels in urine for nicotine and metabolites, while considering both the pharmacological effects of nicotine and a doping perspective which focuses on recent consumption only [33,34]. Thus, in order to ascertain statistical significance for further discrimination between recreational, environmental or doping use, a LLOQ of 10 ng/mL proved to ensure very accurate quantification.

Determination of the best calibration was performed with the evaluation of different curves fitting. Combining accuracy and intermediate fidelity variance allowed building a profile of confidence interval in the dosage range for each target compound [30,31]. According to these accuracy profiles, unweighted linear least-squares regression was found to provide the highest quality results and was chosen for quantification purpose. Due to the linear response, calibration standards were subsequently reduced to LLOQ, medium and ULOQ concentration levels ($k = 3$) and QCs to low, medium and high concentration levels ($k = 3$) with accuracy profiles of comparable quality (Fig. 3). Indeed, accuracy, repeatability and intermediate precision assessments met the guidelines for bioanalytical method validation over the assay range (Table 3). Noteworthy, R^2 corresponding to the initial calibration curve for each compound ($k = 6$) were greater than 0.95, while R^2 with a reduced number of calibrators ($k = 3$) were greater than 0.99. This greatly improved the applicability of this method, allowing a better workflow and simplified calibration.

Therefore, suitability of direct quantification of nicotine and metabolites in urine with this LC–MS/MS method was proven, in particular for nicotine, cotinine and *trans*-3-hydroxycotinine along with nicotine-*N*′-oxide and cotinine-*N*-oxide at concentration ranges of 10–10,000 ng/mL and 10–5000 ng/mL, respectively.

Also, the LOD was found to stand around 500 pg/mL for all compounds.

3.2.2. Selectivity

Selectivity tests towards endogenous matrix compounds were conducted on 6 different urine samples obtained from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days. After extraction in triplicate followed by LC–MS/MS analysis, no interfering endogenous molecules were observed within selected scan windows since ion identification criteria, including retention times, ion transitions and ion ratios, were not met [26].

Likewise, assessment of potential influence of exogenous xenobiotics was performed on a set of over 250 urine samples collected from individuals of the general population who reported joint exposure of nicotine and different substances present in the 2009 Prohibited List and Monitoring Program. Noteworthy, influence of stimulants most commonly found in urine of hockey players was evaluated, among which caffeine and pseudoephedrine. Again, after extraction and LC–MS/MS analysis, no interfering exogenous xenobiotics were observed within selected scan windows according to the criteria mentioned earlier.

3.2.3. Carry-over

Carry-over was evaluated accordingly, after injection of the highest calibrator (10,000 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 5000 ng/mL for nicotine-*N*′-oxide and cotinine-*N*-oxide), followed by the analysis of a blank urine sample. This procedure was repeated three times successively. None of the target compounds were detected, demonstrating the absence of any carry-over effect.

3.2.4. Matrix effect

ME evaluation by comparison of the signals observed in urine and in the neat solution indicated ion enhancement or suppression depending on the target analyte and concentration. Indeed, nicotine, *d*4-nicotine, nicotine-*N*′-oxide and cotinine-*N*-oxide showed significant ion enhancement at both low, medium and high concentrations, while cotinine, *d*3-cotinine, *trans*-3-hydroxycotinine and *d*3-*trans*-3-hydroxycotinine showed substantial ion suppression at low concentration (data not shown). According to the good repeatability of these assessments (RSD < 15%), along with the satisfactory sensitivity and selectivity of the method, ME influence on the results quality was not significant.

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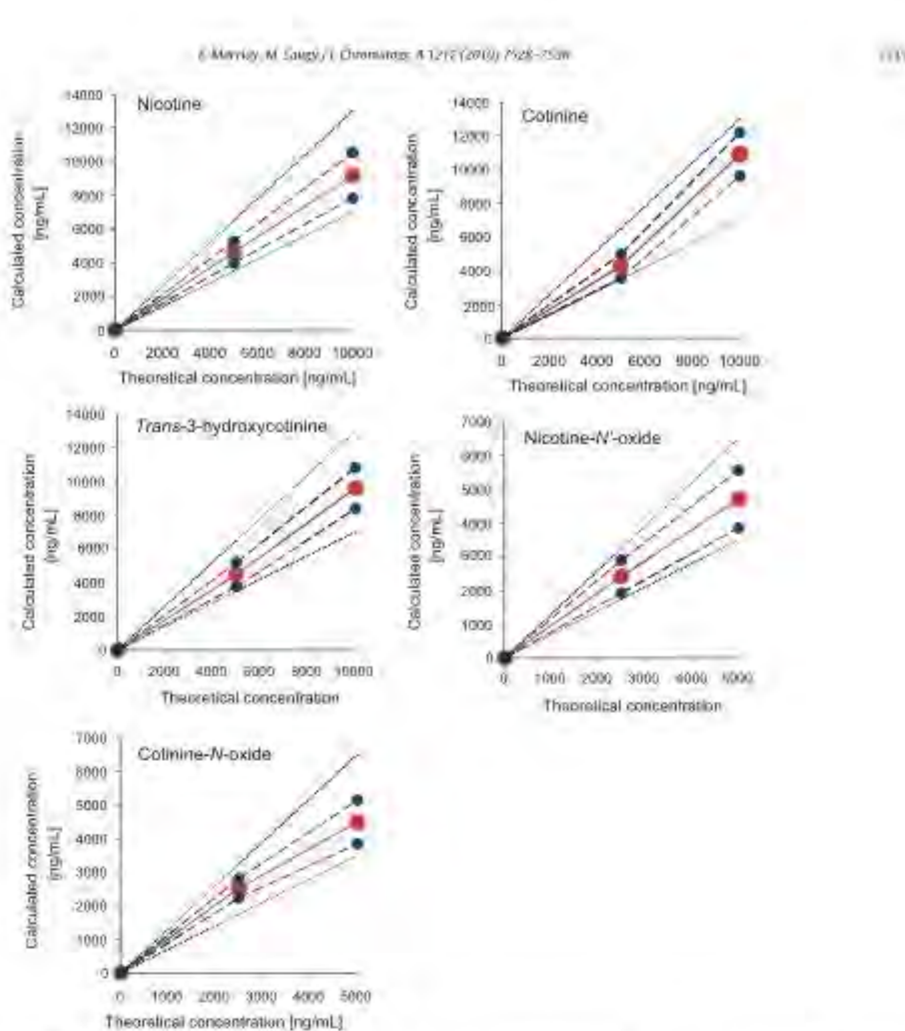

Fig. 3. Absolute accuracy profiles for nicotine and metabolites. The solid line indicates the trueness and the dashed lines represent the accuracy calculated as confidence interval [31]. The dotted lines depict the acceptance limits of ±30%.

3.2.5. Stability

The influence of storage conditions was evaluated by performing a longitudinal stability assay of QC samples every 2 weeks over a period of 6 months. Indeed, these samples experienced freeze and thaw cycles involving successive storage at −20 °C in a complete dark environment and defrost at room temperature, corresponding to storage and analysis conditions during this study.

Referring to the limited variation observed in the peak areas (RSD < 15%), the storage conditions described previously ensured a high stability of all analytes over this particular period of time.

3.3. Application to the IIHF samples

As part of regular doping control protocols during the 2009 IIHF World Championships held in Switzerland, urine samples were collected shortly after every game on two players of each team (n = 72). After approval of the IIHF and Antidoping Switzerland (ADS) and as required by the 2009 International Standards for Laboratories (ISL), article 19 of the World Anti-Doping Code and articles 24–27 of the UNESCO Convention against doping in sport, a minimum storage period of 3 months and complete removal of identification means were ensured prior to use of these samples for research purpose [35–37]. Noteworthy, storage time did not exceed 6 months.

Compounds of interest were quantified in duplicate using a three-point calibration curve together with three urine-based QCs, as described previously. Also, a qualitative value was assigned to metabolites detected in the sub-LLOQ concentration range, namely traces. Concentrations distribution for nicotine and metabolites as quantified in urine specimens are illustrated in Fig. 4. Nicotine, cotinine, trans-3-hydroxycotinine, nicotine-N'-oxide and cotinine-N-oxide concentrations ranged between 11 and 19,750, 13 and 10,475, 10 and 8217, 11 and 3396, and 13 and 1640 ng/mL, respectively.

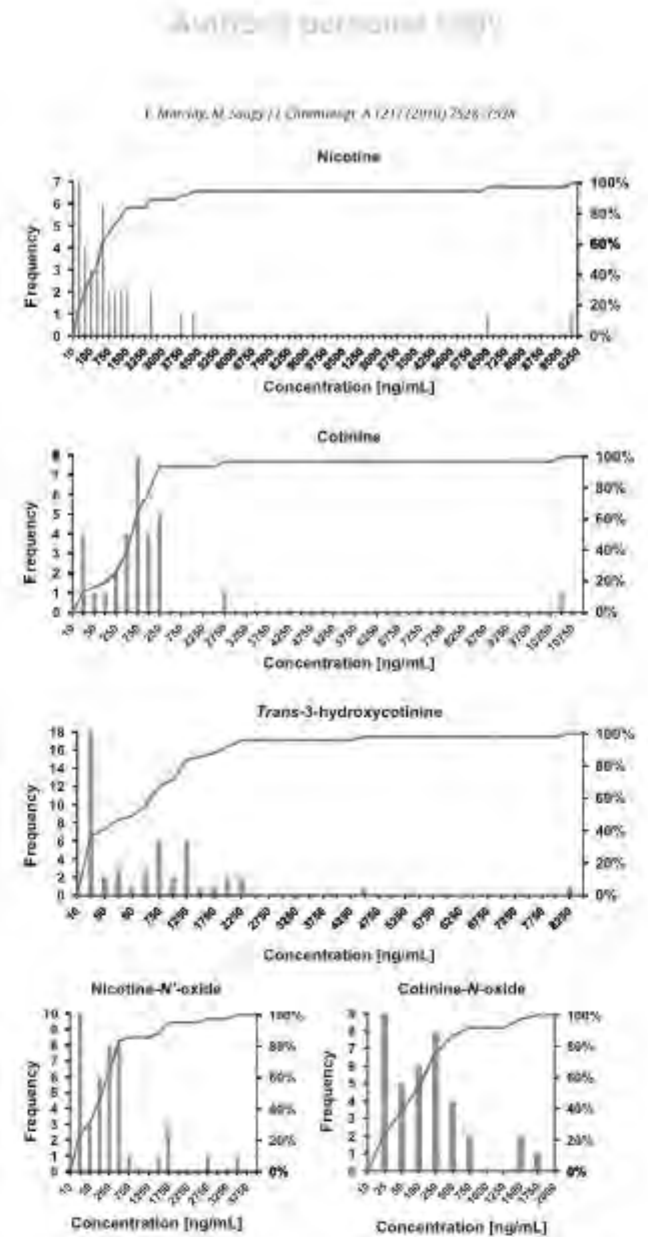

Fig. 4. Concentrations distribution for nicotine and metabolites. The solid line indicates the cumulative percentage.

Traces of nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide were detected in 87, 91, 94, 97 and 97% of samples, respectively (Table 4). Noteworthy, at least one of the five different metabolites was present in every sample. These findings suggest that every athlete was exposed to nicotine, either environmentally or from active consumption, during the competition period. Such results should be carefully interpreted regarding prevalence studies on smoking in society and on environmental tobacco exposure (ETS) among non-smokers. Indeed, smoking prevalence has been reported by the World Health Organization (WHO) as ranging from 15 to 44% depending on the country participating to the 2009 IIHF World Championships [38]. Also, ETS for a period of at least 1 h per day reached 21% in Switzerland, which hosted the competition [39]. However, both facts may explain only parts of such extensive nicotine exposure, especially when considering that athletes are significantly less likely to smoke or be exposed to smoke than the general population.

Furthermore, above LLOQ levels of the previously mentioned compounds were measured in 51, 43, 68, 58 and 51% of samples, respectively. One of the five different metabolites was detected at

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Table 4
Prevalence of IIHF urine samples exposed to nicotine or/and metabolites depending on the concentration range (n = 72).

Analyte	Concentration range		
	LOD ≤ x ≤ LLOQ	LLOQ ≤ x ≤ ULOQ	Active exposure
Nicotine	47.5%	51.4%	36.1%
Cotinine	95.7%	43.1%	39.1%
trans-3-Hydroxycotinine	94.4%	68.1%	40.2%
Nicotine-*N'*-oxide	97.2%	58.3%	44.4%
Cotinine-*N*-oxide	97.2%	51.4%	38.9%
Summarized exposure	100.0%	83.3%	52.7%

such concentrations in 82% of samples. These results also indicate that exposure was within the last 3 days previous to the games for approximately 8 ice hockey players out of 10 [40].

Prevalence of nicotine consumption, in the form of smoke or smokeless nicotine, close to or/and during the games was evaluated by hypothesizing conservative concentration limits for active consumption (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-*N'*-oxide and cotinine-*N*-oxide) [40]. Also, chances of exposure to serious environmental smoke within the few hours prior to games of such importance were excluded. Thus, according to these concentration limits, active nicotine use was highlighted in 36–44% of samples, depending on the target compound (Table 3). Noteworthy, at least one of the five different metabolites was present at such levels in 53% of the urine samples, emphasizing a significant prevalence of nicotine consumption amongst ice hockey players.

Interestingly, two samples presented highly elevated nicotine concentrations exceeding the upper limit of quantification (ULOQ). Such acute exposure to nicotine is hardly achievable for a regular consumer [34,41] thus significantly supporting the likelihood of use for doping purpose. Also, since nicotine half-life is relatively short, consumption close to or/and during the game is the most likely hypothesis. Thus, according to the quantitative measurements performed and the detrimental respiratory effects due to extensive smoking prior to sport practice [42–45], sound evidence on smokeless nicotine use may be hypothesized for these two samples. However, due to the lack of clinical studies addressing metabolic-based distinction between different forms of nicotine consumption, such conclusions could not be extended to other samples. Likewise, specific studies addressing the relationship between nicotine levels and doping are missing, hence the careful assumptions made here.

4. Conclusion

A sensitive and selective HILIC–ESI-MS/MS method for the simultaneous detection and quantification of nicotine and its four principal metabolites in urine was developed and fully validated. The simple and fast sample preparation protocol based on LLE provided a satisfactory matrix clean-up and recovery, while the subsequent use of hydrophilic interaction chromatography allowed to obtain very good separation and peak shape, enhanced sensitivity and high samples throughput.

This analytical procedure was successfully applied to the urine samples collected during the 2009 Ice Hockey World Championships, in order to investigate the prevalence of nicotine consumption amongst athletes. The findings gathered during this work provided strong evidence that nicotine is a very serious trend in ice hockey. Indeed, traces of nicotine or metabolites were found in every urine sample, with concentration levels corresponding to exposure within the last 3 days for approximately eight specimens out of ten. Prevalence of nicotine consumption, in the form of smoke or smokeless nicotine products, before or/and during the games suggested that about half of the ice hockey players were active users. Noteworthy, highly elevated nicotine concentrations were measured in two samples, significantly supporting the likelihood of use of smokeless tobacco for doping purpose.

Assuming that smoking and sport practice at top level are not compatible, these results give a strong indication on the use of smokeless nicotine in ice hockey. Thus, nicotine consumption in ice hockey is a very serious phenomenon which requires further investigation on its use as a potential doping agent.

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Brief Report

Assessing Tobacco Dependence Among Cannabis Users Smoking Cigarettes

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Abstract

Introduction: This study examines the relationship between nicotine exposure and tobacco addiction among young smokers consuming either only tobacco or only tobacco and cannabis.

Methods: Data on tobacco and cannabis use were collected by a questionnaire among 313 adolescents and young adults in Western Switzerland between 2009 and 2010. In addition, a urine sample was used to determine urinary cotinine level. Nicotine addiction was measured using the Fagerström Test for Nicotine Dependence (FTND). In this study, we focused on a sample of 142 participants (mean age 19.54) that reported either smoking only tobacco cigarettes (CIG group, $n = 70$) or smoking both tobacco cigarettes and cannabis (CCS group, $n = 72$).

Results: The FTND did not differ significantly between CIG (1.96 ± 0.26) and CCS (2.66 ± 0.26) groups ($p = 0.07$). However, participants in the CCS group smoked more cigarettes (8.30 ± 0.79 vs. 5.78 ± 0.8, $p = 0.03$) and had a higher mean cotinine value (671.18 ± 67.6 vs. 404.32 ± 68.63, $p = 0.008$) than the CIG group. Further, the association between cotinine and FTND was much stronger among the CIG than among the CCS group (regression coefficient of 0.0031 vs. 0.00099, $p < 0.0001$).

Conclusion: Adolescents smoking tobacco and cannabis cigarettes featured higher levels of cotinine than youth smoking only tobacco; however, there was no significant difference in the addiction score. The FTND score is intended to measure nicotine dependence from smoked tobacco cigarettes. Hence, to accurately determine nicotine exposure and the associated dependence among young smokers, it seems necessary to inquire about cannabis consumption.

Introduction

Cannabis is the most common illicit drug used in Switzerland, and most consumers begin to use it during adolescence. In the 2010 Health Behavior in School-aged Children study, almost 30% of 15-year-old Swiss pupils had ever consumed cannabis and 7% of them reported consuming cannabis on a regular basis (defined as the use of cannabis at least three times during the last 30 days; Windlin, Delgrande, & Kuntsche, 2011).

Cannabis use and tobacco smoking are strongly linked and the majority of cannabis users also smoke tobacco (Leatherdale, Hammond, Kaiserman, & Ahmed, 2007). The concomitant use of cannabis and tobacco is of concern because cannabis use has been found to promote the transition to more intensive tobacco smoking (Agrawal, Madden, et al., 2008) and to make quitting tobacco smoking more difficult (Amos, Wiltshire, Bostock, Haw, & McNeill, 2004; Ford, Vu, & Anthony, 2002). Further, it has been reported that cannabis use during adolescence or young adulthood may increase the risk of developing later nicotine dependence (Patton, Coffey, Carlin, Sawyer, & Lynskey, 2005; Timberlake et al., 2007). One study examined the influence of the concomitant use of tobacco with cannabis on nicotine dependence and found that users of both substances were more likely to be nicotine dependent than noncannabis users (Okoli, Richardson, Ratner, & Johnson, 2008).

There are many theories trying to explain this increased risk for tobacco dependence among cannabis users. Physiologically, it has been demonstrated that knock-out mice not expressing cannabinoid receptors differed from wild-type mice in some behavioral responses induced by nicotine (Castane, Valjent, Ledent, Parmentier, Maldonado, & Valverde, 2002). This suggests a central interaction between the cannabinoid system and nicotine. Further, twin studies have evaluated the genetic risks underlying the use of licit and illicit drugs, and a common genetic predisposition was found for cannabis and nicotine use (Agrawal, Lynskey et al., 2008; Young, Rhee, Stallings, Corley, s& Hewitt, 2006).

Tobacco is commonly added to cannabis to reduce its strength, make it burn better and reduce the consumption costs (Akre, Michaud, Berchtold, & Suris, 2010). This process, referred as mulling, is frequent among cannabis users particularly in the

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Assessing tobacco dependence among cannabis users smoking cigarettes

European countries where cannabis is most often smoked as joints (cannabis cigarettes; Belanger, Akre, Kuntsche, Gmel, & Suris, 2011). Mulling may thus explain an intensified propensity for tobacco dependence through increased nicotine exposure. To our knowledge, no study has yet examined that possibility.

Overall the use of cannabis among adolescents continues to be a major health issue not only because of its related physical and psychological adverse effects (Hall & Degenhardt, 2009) but also due to its association with cigarette initiation, nicotine dependence, and difficulties to quit smoking.

The objectives of this study are thus to compare nicotine dependence among both young people concurrently smoking cannabis and cigarettes and those smoking only cigarettes, as well as to correlate it with their total nicotine exposure. Our hypothesis is that because of mulling, nicotine dependence scores will be higher among those also smoking cannabis, mainly from their increased nicotine exposure.

Methods

We used data on tobacco and cannabis consumption that were collected between 2009 and 2010 in Western Switzerland (Bélanger et al., 2010). About 313 participants aged 16–25 were recruited among the general population by the means of advertisement in public places, on the Internet and from participating clinics. A snowball technique was also used where participants invited their peers to get involved in the study. People were assigned to one of four groups according to their smoking status: nonsmokers, cigarette-only smokers, cannabis-only smokers, and smokers of both cigarettes and cannabis.

Participants filled in an anonymous questionnaire and a urine sample was collected to blindly measure its cotinine level using liquid chromatography coupled mass spectrometry. Cotinine is a highly specific and sensitive indicator of tobacco use, and it has been demonstrated that its concentration correlates well with nicotine exposure (Bramer & Kallungal, 2003).

Nicotine addiction was measured with the Fagerstrom Test for Nicotine Dependence (FTND), which consists of six questions. The FTND score ranges from 0 to 10 with higher scores meaning a greater dependence (Fagerstrom, Heatherton, & Kozlowski, 1990). Tobacco use was assessed by the questions "How many cigarettes did you smoke yesterday?" and "How many cigarettes did you smoke during the last five days?". To determine the quantity of tobacco added to cannabis cigarettes ("mulling"), participants were asked to indicate the proportion of cannabis and tobacco they put in the joints or blunts they smoked. The complete methodology of this study has been described elsewhere (Bélanger et al., 2010).

In this study, we focused on participants reporting either smoking only cigarettes or smoking both cigarettes and cannabis. Those having incomplete information on active or passive tobacco exposure, actively exposing themselves to tobacco in the previous five days other than through cigarettes or mulling, or reporting cannabis use without adding tobacco to it had to be excluded of the current analysis.

Differences between the two groups on selected variables were assessed with Students t tests (continuous variables) or chi-square tests (categorical variables). To determine the association between the mean cotinine values and the FTND scores, we computed independently for each group a linear regression using FTND as the dependent variable and adjusting for age and sex. Finally, we tested the difference between the two regression slopes still controlling for age and sex. Stata 12 (StataCorp, College Station, TX) was used for all statistical computations.

Results

The sample included 142 subjects (mean age 19.54) with 59.15% being females. This sample was divided between the cigarette-only smoking group (CIG, $n = 70$; 77.14% females) and the cigarette and cannabis smoking group (CCS, $n = 72$, 41.67% females). The difference in female proportion was significant between the two groups ($p < 0.001$). There was no significant difference in the mean FTND score between the CIG (1.98 ± 0.26) and the CCS (2.66 ± 0.26) group ($p = 0.07$) (analysis adjusted for gender). In a post-hoc analysis, there was no significant difference between the two groups when looking at the six items of the FTND score separately (data not shown).

The CCS group smoked significantly more cigarettes than the CIG group (8.30 ± 0.79 vs. 5.78 ± 0.80 cigarettes smoked the day before, $p = 0.03$; and 43.26 ± 3.59 vs. 28.48 ± 3.65 cigarettes smoked during the last five days, $p = 0.006$). However, it is worth noting that both reports of cigarettes smoked per day yield identical scores on the FTND (where smoking 10 or less cigarettes per day yields a score of 0). The CCS group also showed a higher mean cotinine value than the CIG group (671.18 ± 67.60 ng/ml vs. 404.32 ± 68.63 ng/ml, $p = 0.008$). Regarding the consumption of cannabis, participants from the CCS group smoked 2.48 ± 0.20 cannabis cigarettes the day before and 8.10 ± 0.75 during the last five days (Table 1).

The cotinine concentration was positively correlated with the FTND score. When looking at each group separately, the association between cotinine and FTND was much stronger among the CIG group (regression coefficient of 0.0031, $p < 0.001$) than among the CCS group (regression coefficient of 0.00099, $p < 0.0001$) and the two regression slopes were significantly different ($p < 0.0001$; Figure 1).

Our survey was not primarily designed to assess differences in the FTND, and this can explain the low statistical power (33%). However, to go a step further, we conducted a bootstrap analysis using 10,000 replicated samples. Results showed that the difference between groups remained nonsignificant ($p = 0.06$). Even if this analysis does not constitute an absolute proof, it clearly diminished the potential limitations associated with our statistical power. Future research with larger sample sizes is needed to confirm our findings. In addition, because the FTND is not exactly normally distributed among the two groups, the result of the t-test was double-checked using the Wilcoxon rank-sum test, but the results did not change ($p = 0.07$). This was also confirmed by bootstrap ($p = 0.117$).

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Table 1. Characteristics of Smokers' Groups

	Total (N = 142)	Cigarette smokers (n = 70)	Cigarette + cannabis smokers (n = 72)	p value
Gender (% females)	59.15	77.14	41.67	<0.001
Mean age (years)	19.54 ± 2.40	19.23 ± 2.38	19.85 ± 2.40	0.13
Mean urinary cotinine (ng/ml)[a]	545.16 ± 47.64	404.32 ± 68.63	873.13 ± 67.60	0.008
Mean number of cigarettes smoked the day before[a]	7.07 ± 0.55	5.78 ± 0.80	8.30 ± 0.79	0.03
Mean number of cigarettes smoked during the last five days[a]	35.74 ± 2.54	28.48 ± 3.65	43.26 ± 3.59	0.006
Mean number of cannabis cigarettes smoked the day before (n = 55)[a]	—	—	2.48 ± 0.20	—
Mean number of cannabis cigarettes smoked during the last five days[a]	—	—	8.10 ± 0.75	—
Mean Fagerstrom score[a]	2.31 ± 0.18	1.96 ± 0.26	2.66 ± 0.26	0.07

[a]Adjusted for gender.

Discussion

We found no significant difference in the mean tobacco addiction score as measured by the FTND between adolescents smoking cigarettes and those smoking cannabis and cigarettes. We neither found differences between the two groups for each of the different items forming the FTND.

Prior research has shown that the FTND is associated with urine cotinine (Carpenter, Baker, Gray, & Upadhyaya, 2010; Etter, Vu Duc, & Perneger, 2000) and that a correlation could be demonstrated even at low cotinine levels (Rubinstein, Thompson, Benowitz, Shiffman, & Moscicki, 2007). When looking at this relationship among each group, we observed that the association between the FTND and cotinine was much stronger in the CIG than in the CCS group. Hence, the addiction score among the CCS group did not reflect the real nicotine exposure. In the same line, adolescents in the CCS group smoked more cigarettes per day, which may explain a slightly higher cotinine level. However, this difference in the number of cigarettes smoked is not reflected in the FTND score.

The large difference in the cotinine values between groups may be explained by the phenomenon of mulling. All participants of the CCS group in the current analysis declared smoking cannabis in which tobacco had been added, with the majority of them estimating the proportion of tobacco in their cannabis cigarettes to be between 25% and 50% (Bélanger et al., 2010). However, a limitation is that we did not know the quantity of tobacco that was effectively used in the cannabis cigarettes.

The difference in cotinine level may alternatively be explained by the difference in the way cannabis cigarettes are smoked compared to tobacco cigarettes. The average volume of puffs has been found to be much larger and the duration of puffs much longer when smoking a cannabis cigarette (Wu, Tashkin, Djahed, & Rose, 1988). As the puff volume of a tobacco cigarette is correlated to the nicotine blood level (Herning, Jones, Benowitz, & Mines, 1983), a moderate quantity of tobacco mixed in a cannabis cigarette may induce an important quantity of nicotine. Another potential explanation may be a difference in the metabolism of nicotine between the CIG and the CCS groups, a theory that might be worth exploring in future studies.

Unlike our results, a prior cross-sectional study conducted among adolescents (Okoli et al., 2008) found higher scores of perceived and measured tobacco dependence among cigarette smokers using cannabis than among those not using cannabis. This discrepancy with our results may be explained by their use of the modified Fagerström Tolerance Questionnaire for adolescents who may be more sensitive for moderate smokers. We used the adult version of the FTND that we found more adapted to the age range of our study population (young adults) than the modified version for adolescents. Further, studies assessing

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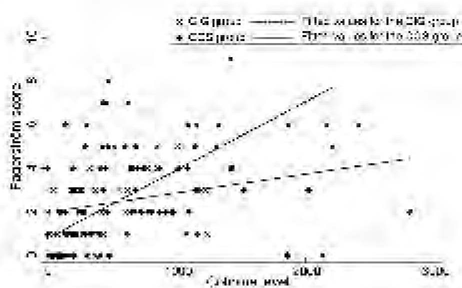

Figure 1. Relationship between urinary cotinine and Fagerström score.

the influence of cannabis use on tobacco dependence should report how cannabis was used as it has been demonstrated that tobacco dependence varied according to how it is consumed (Timberlake, 2009). In our study, the FTND scores did not reflect the differences in nicotine exposure. Yet the FTND is meant to measure nicotine dependence from tobacco cigarettes only. If a smoker meets his nicotine need with a cannabis cigarette containing tobacco, he will not score it on the FTND. As an example, if one usually starts his day smoking a cannabis cigarette containing tobacco, he will not report smoking a cigarette after waking up.

The strength of our study is that we combined a score of tobacco addiction with an objective measure of nicotine exposure, the urinary cotinine level. The main limitations of our study are the cross-sectional nature of our data preventing us to assess causality, the convenience sample, and the small sample size that restricts the generalization of the results. Further, data were collected using a questionnaire, which makes them susceptible to recall bias. However, the recall period was only of five days, which reduces the probability of bias.

Hence, youth smoking cigarettes and cannabis may have a high exposure to nicotine, which may be underestimated when nicotine dependence is assessed by the FTND. As it has already been concluded previously (Highet, 2004), when investigating the nicotine addiction experienced by young smokers, health care professionals should not only ask about the quantity of cigarettes smoked but also inquire about the consumption of cannabis and the way it is consumed. This may be of importance in the clinical setting when an adolescent consuming both tobacco cigarettes and cannabis cigarettes mixed with tobacco wishes to quit smoking as his nicotine need during replacement therapy will be higher than that of a youth smoking only tobacco.

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Declaration of Interests

None declared.

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ORIGINAL INVESTIGATION

To What Extent Does Adding Tobacco to Cannabis Expose Young Users to Nicotine?

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ABSTRACT

Introduction: To determine if mulling, the process of adding tobacco to cannabis for its consumption, exposes young cannabis users to significant levels of nicotine.

Methods: This observational study performed in 2009–2010 among Swiss youths aged 16–25 involved the completion of a self-administrated questionnaire and the collection of a urine sample on the same day. Measures of urinary cotinine were blindly performed using liquid chromatography coupled–mass spectrometry. A total of 197 eligible participants were divided in 3 groups based on their consumption profile in the past 5 days: 70 abstainers (ABS) not having used cigarettes or cannabis, 57 cannabis users adding tobacco to the cannabis they smoke (MUL) but not having smoked cigarettes, and 70 cigarette smokers (CIG) not having smoked cannabis.

Results: Exposure to nicotine was at its lowest among ABS with a mean (*SE*) cotinine level of 3.2 (1.4) ng/ml compared, respectively, with 214.6 (43.8) and 397.9 (57.4) for MUL and CIG ($p < .001$). While consumption profile appeared as the only significant factor of influence when examining nicotine exposure from the ABS and MUL participants on multivariate analysis, it did not result in substantial differences among MUL and CIG groups.

Conclusions: Urinary cotinine levels found among MUL are high enough to indicate a significant exposure to nicotine originating from the mulling process. In line with our results, health professionals should pay attention to mulling as it is likely to influence cannabis and cigarette use, as well as the efficacy of cessation interventions.

INTRODUCTION

Confusion has recently emerged on what the word "smoking" really relates to. Formerly used only for cigarettes and other tobacco products, it now also refers to cannabis use. While both substances share inhalation as the preferred way of consumption (Agrawal, Madden, Bucholz, Heath, & Lynskey, 2008; Akre, Michaud, Berchtold, & Suris, 2010), this drift may originate from the vast number of adolescents and young adults who use cannabis nowadays. According to the 2006 Health Behaviour in School-aged Children (HBSC) survey, more than one tenth of adolescents aged 15 living either in the United States, Canada, or in European countries such as Spain and Switzerland, use cannabis at least monthly, and nearly one third have tried the substance at least once (Currie et al., 2008).

Cigarette smoking still typically precedes cannabis use. For this reason, it has been considered for more than 35 years a first necessary step, with alcohol, to the use of illegal substances (Kandel, 1975). Yet, this sequence is less and less the rule. It is currently up to one fifth of young cannabis users having never smoked a cigarette (Suris, Akre, Berchtold, Jeannin, & Michaud, 2007) or at least not in the previous month (Degenhardt et al., 2010). Weekly cannabis use among such cigarette abstainers seems to convey more than an eightfold increase in the odds of later tobacco initiation (Patton, Coffey, Carlin, Sawyer, & Lynskey, 2005). Young cannabis users already smoking cigarettes seem even more likely to transition to higher stages of smoking later on (Agrawal et al., 2008; Patton et al., 2005). As a result, evidences supporting a reverse gateway phenomenon suggest that nicotine dependence and persistent cigarette smoking may in fact be the leading and most alarming public health consequence of cannabis use (Agrawal et al., 2008). Despite cannabis being linked to several adverse health problems (Hall & Degenhardt, 2009), tobacco is still the number one cause of preventable mortality in the world (World Health Organization, 2009).

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A growing number of studies report that tobacco is commonly added to cannabis for its consumption (Akre et al., 2010; Amos, Wiltshire, Bostock, Haw, & McNeill, 2004; Belanger, Akre, Kuntsche, Gmel, & Suris, 2011; Patton et al., 2005). This process of adding tobacco to cannabis, further referred as mulling, seems mainly performed because pure cannabis cigarettes (joints) are too strong, too expensive, and do not burn correctly (Akre et al., 2010). Although this process is believed to be rather exceptional in the United States compared with blunt smoking (Ream, Benoit, Johnson, & Dunlap, 2008), quantitative and qualitative studies performed in Switzerland and the United Kingdom (Akre et al., 2010; Amos et al., 2004; Belanger et al., 2011), as well as anecdotal reports originating from Australia and Canada, describe mulling as widespread (Leatherdale, Ahmed, & Kaiserman, 2006; Patton et al., 2005). In Switzerland, it is four of every five young cannabis users who report this process to be frequently performed (Belanger et al., 2011).

Even if inference may be drawn that nicotine exposure originates from mulling, no study has ever established to which extent cannabis users expose themselves to tobacco smoke through this process. Such information would help determine if mulling is a key factor in explaining the reverse-gateway phenomenon, how it may add to symptoms of cannabis dependence, as well as interfere with the treatment of both substances. The primary objective of this study was thus to determine if mulling exposes cannabis users to significant levels of nicotine, well above possible environmental exposure. Secondarily, we were interested in comparing the level of nicotine exposure from mulling to the level from cigarette smoking alone.

METHODS

This research was part of a larger cross-sectional observational study of young cannabis users having been approved by the Ethics Committee of the University of Lausanne School of Medicine, Switzerland (Belanger et al., 2010). Recruitment of participants took place over a 10-months period, from November 2009 to September 2010. Adolescents and young adults from the community, regardless of their substance use, were mainly invited to participate through postal and online ads. Participants were specifically asked to tell friends about the study as a way to increase possible enrolment. More than 350 youths were fully screened for 3 inclusion criteria: being aged between 16 and 25, not having a chronic condition that could interfere with the metabolism of either cannabis or nicotine, and not being pregnant. If enrolled, participants completed a brief questionnaire and provided a urine sample on a single visit. Signed consent from minors was accepted as all participants were above 16 years old in accordance with national consent standards for the study design. Participants were given a unique code to contact the research group to get their results if wanted and received a 25 Swiss Francs (~25 US$) gift card from a department store.

Assessment of Cannabis and Tobacco Use and Passive Exposure to Tobacco

A preliminary questionnaire was built and revised by several adolescents to ascertain its understanding. The final questionnaire included 32 questions (103 items) that covered personal characteristics and substance use (Belanger et al., 2010). A comprehensive assessment of tobacco use over the last 5 days was performed using questions on cigarette smoking, as well as on the use of other tobacco products. Similar questions also inquired cannabis use (number of times a user took part in cannabis consumption, i.e., whether one whole cannabis cigarette or only one puff). Regarding the mulling process, participants were asked how many cannabis cigarettes/cigars they smoked contained tobacco, and in which proportion cannabis and tobacco was usually mixed. Questions mainly inquired consumption on the day of their visit, on the prior day, and over the previous 5 days. Passive exposure to tobacco was determined using questions from the works of Nondahl, Cruickshanks, and Schubert (2005) covering exposure at home, at work (further adapted to cover both work and school), and in social settings. Answers were then dichotomized for each of the three passive exposure sources into the lowest level versus the others.

Biochemical Analysis

In accordance with the literature, urinary cotinine was chosen as a valid and convenient marker for both active and passive exposure to tobacco smoke (Bramer & Kallungal, 2003). Cotinine is the main primary metabolite of nicotine and its urinary half life is of 18–20 hr (Matsuki et al., 2008; Schepis et al., 2008). As a result, urine cotinine concentrations usually decline to nonsmoking levels within 3–4 days (Bramer & Kallungal, 2003). Inquiring participants on their tobacco consumption in the prior 5 days of their visit thus provides a suitable way to firmly establish from which source nicotine exposure ensued. Each urine sample was transferred to a transport syringe of 10 ml (Monovette®, SARSTEDT AG & Co) and then refrigerated for a maximum of 48 hr before being frozen at −20 °C. Measures of urinary cotinine (nanogram per milliliter) were blindly performed using liquid chromatography coupled–mass spectrometry (Marclay & Saugy, 2010).

Sample

Overall, 313 individuals were met and gave consent to participate. Based on the questionnaire, four groups of participants were created: abstainers (ABS—not having used cigarettes or cannabis in the previous 5 days), cannabis smokers adding tobacco to the cannabis they smoke (MUL—having smoked cannabis in which tobacco had been added, but not cigarettes during the same period), cigarettes smokers (CIG—having smoked cigarettes but not cannabis), and cannabis and cigarette smokers (CCS—having smoked both). Overall, 44 participants did not met the criteria to get assigned to one of these groups. Some had incomplete information on active or passive tobacco exposure ($n = 2$), or reported cannabis use without adding tobacco to it ($n = 3$), and 39 actively exposed themselves to tobacco in the previous 5 days other than through cigarettes or mulling (i.e., Snus, Snuff, Cigar, and Chicha), which prevented us from distinguishing their nicotine exposure origin. Participants from the CCS group ($n = 72$) were excluded from the present analysis because they were exposed to tobacco through both cigarette smoking and mulling in the past 5 days. Accordingly, data from 70 ABS, 57 MUL, and 70 CIG were used.

Statistical Analysis

Participants' characteristics were described using means and proportions, while differences between groups were identified

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using single factor analysis of variance. Cotinine levels of participants were first analyzed according to their consumption group. To provide useful information about the nicotine exposure of each study group, cotinine levels were benchmarked to known cotinine standards that differentiate smokers on heaviness of smoking, including light smokers (50–150 ng/ml), moderate smokers (151–600 ng/ml), and heavy smokers (>600 ng/ml) (Galanti, 2008; SRNT Subcommittee on Biochemical Verification, 2002). Afterward, the urinary level of cotinine among participants from the ABS and MUL groups were compared to determine if mulling represented a significant exposure to nicotine. Likewise, participants from CIG and MUL groups were compared on their cotinine levels to determine to which extent mulling may differ from cigarette smoking. Multiple linear regressions were used, controlling for age, gender, body mass index (BMI: kg/m^2) based on self-reported height and weight, ethnicity (caucasian vs. other), and passive exposure to cigarette smoke to account for known confounders (Hukkanen, Jacob, & Benowitz, 2005). All variables were entered simultaneously in the regression model. The analysis between the CIG and MUL groups additionally took into account the latest (hours) consumption of tobacco (either from cigarettes or mulling). Finally, to gain a sense of which cigarette smoking group the mulling group was most comparable to, we performed ad-hoc analysis between subjects belonging to the MUL group and three CIG subgroups according to their cigarette consumption in the prior 5 days (≤20, 21–60, and >60 cigarettes). In every linear regression, MUL was compared with the reference group. Stata 11 (StataCorp LP) was used for all statistical computations. Even if a total 70 individuals per group were first sought to achieve our main objective (more details can be found in Bélanger et al., 2010), a posteriori computations showed that the reduction in power induced by the smaller sample size in one of the three groups was only marginal to achieve a power at least equal to 80% for the comparisons.

RESULTS

Participants' characteristics are reported in Table 1. Most notably, participants from the MUL group were mostly males, whereas those in the ABS and CIG groups were predominantly females.

Over the 5 days prior to their visit, participants in the CIG group had smoked on average 30.5 cigarettes (Table 2). For most (68.6%), last consumption occurred the same day of their visit, with 89.6% of them having smoked in the hour prior to their participation. During the same 5 days, 93.7% of cannabis consumptions reported by the MUL group included tobacco. Based only on cannabis consumptions in which tobacco had been added, MUL had used cannabis on average 8.8 times in the past 5 days, with at least one consumption the exact day of the visit for 24.6% of them.

Among participants from the MUL group, 35% reported blending one-fourth of tobacco to three-fourth cannabis, and 30% reported an equal mix. Adding three-fourth of tobacco to one-fourth of cannabis was reported less frequently (12%). Intermediate mixing proportions were reported by 16% of participants (6 participants reporting tobacco proportions ranging from 30% to 40%, and 3 from 60% to 70%). Only 7% of participants in the MUL group did not know the proportion of tobacco being usually added to the cannabis they smoked.

Table 1. Characteristics of Participants

	ABS (n = 70)	MUL (n = 57)	CIG (n = 70)	p value
Gender: Male (n)	32.9% (23)	70.2% (40)	22.9% (16)	<.001
Age: mean (SE)	19.6 (0.29)	19.8 (0.30)	19.2 (0.28)	.370
Ethnicity: Caucasian (n)	88.6% (62)	96.5% (55)	91.4% (64)	.266
BMI: mean (SE)	20.9 (0.28)	21.8 (0.43)	22.1 (0.49)	.091

Note. BMI = body mass index.
BMI is based on self-reported height and weight. The last column provides the p value of the analysis of variance comparing the three groups.

Table 2. Substance Use of Participants Over the 5 Days Prior to Visit and Urinary Cotinine Level

	ABS		MUL		CIG
Mean consumption[a] (SE)	–		8.8 (8.4)		30.5 (29.4)
Consumption distribution[a] (n)					
	–	Up to 5	47.4% (27)	Up to 20	51.5% (36)
	–	5–10	22.8% (13)	21–60	31.4% (22)
		More than 10	29.8% (17)	More than 60	17.1% (12)
Last consumption (n)					
Same day of visit	–		24.6% (14)		68.6% (48)
The day prior to visit	–		43.8% (25)		14.3% (10)
Two to 5 days prior to visit	–		31.6% (18)		17.1% (12)
Mean urinary cotinine level[b] (SE)	3.2 (1.4)		214.6 (43.8)		397.9 (57.4)

Note. [a]Numbers reported for MUL are cannabis consumptions where tobacco had been added, while for CIG, cigarettes smoked.
[b]Urinary cotinine level (nanogram/milliliter).

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Adding tobacco to cannabis expose young users to nicotine

Nicotine exposure differed vastly according to consumption groups (Table 2). Exposure was at its lowest among ABS with a mean (*SE*) of 3.2 (1.4) ng/ml of cotinine found in their urine samples. Individuals from the MUL group had a mean cotinine level of 214.6 (43.8) ng/ml and those in the CIG group of 397.9 (57.4) ng/ml. All differences between groups were highly significant ($p < .001$) on univariate analysis.

As seen in Figure 1, comparing with accepted cigarette exposure standards, almost all of samples from ABS (98.6%) revealed cotinine levels in the range of non-tobacco users. In contrast, the majority of cotinine measures from participants in the MUL group (56.2%) was above 50 ng/ml, with more than one third of their samples showing cotinine levels at least comparable with standards for moderate cigarette smokers. For most of the participants in the CIG group, cotinine levels extended over the level of moderate cigarette smokers (58.6%).

In the first multivariate analysis (Table 3), being part of either the ABS or the MUL group was the only variable significantly associated with the cotinine level of participants ($p < .001$). In contrast, consumption profile (being part of one group over the other) did not appear as a significant variable for cotinine levels when examining MUL and CIG participants. In fact, only time since last consumption did. Accordingly, cotinine levels between participants from the MUL and CIG groups were not significantly different in the multivariate regression, accounting for possible confounders. When focusing only on participants from the CIG subgroup smoking more than 60 cigarettes in the previous 5 days, the MUL group showed a significantly lower urinary cotinine level ($p < .001$). Additionally, a gender difference appeared when comparing participants smoking between 21 and 60 cigarettes per day with the MUL group.

DISCUSSION

Two main findings originate from this observational study on nicotine exposure. First, the urinary cotinine levels found among cannabis users not smoking cigarettes (MUL) are high enough to indicate a significant exposure to nicotine originating from the mulling process. Second, nicotine exposure to most cannabis users not smoking cigarettes from our study seems comparable with standards observed among light and moderate cigarette smokers.

Since mulling is highly common among cannabis users in Switzerland (Belanger et al., 2011), having found elevated cotinine levels in a subgroup of these substance users not smoking cigarettes is not surprising. Yet, we did not expect to find such high levels. In fact, most cannabis consumption is known to take place in groups where cannabis cigarettes are usually shared, consequently decreasing the tobacco exposure from a single consumption. Moreover, participants from the MUL group reported far less frequent cannabis consumptions than cigarettes smoked by CIG participants, and the proportion of tobacco being added to cannabis seems to reach a maximum of 50% for most users. Nonetheless, such mixing proportion may not be insignificant. As cannabis smoking involves more profound inhalation with longer puffs than for cigarettes (Wu, Tashkin, Djahed, & Rose, 1988), this could result in higher exposure to nicotine from the tobacco being added. Works by Van Der Kooy, Pomahacova, and Verpoorte (2008) have already demonstrated that this specific topography of cannabis smoking (deeper and longer inhalation) resulted in higher exposure to tetrahydrocannabinol (THC) from cannabis cigarettes compared with a more superficial way to smoke, as this is the case for cigarettes.

An increased extraction of nicotine during the combustion of the tobacco/cannabis blend could be another explanation for the elevated cotinine levels found among MUL participants. In accordance, using the right mixing proportions, THC extraction can be increased up to 50% when tobacco is added to a cannabis cigarette (Van der Kooy, Pomahacova, & Verpoorte, 2008). However, for THC, it seems to result from a higher temperature being reached in a cannabis cigarette by the addition of tobacco rather than through a particular chemical reaction.

Our finding may also originate from an altered metabolism of nicotine, increasing its half-life, from the coinhalation of THC or its metabolites. Yet, one study performed in 1988 described mean levels of cotinine in the plasma of cannabis and cigarette

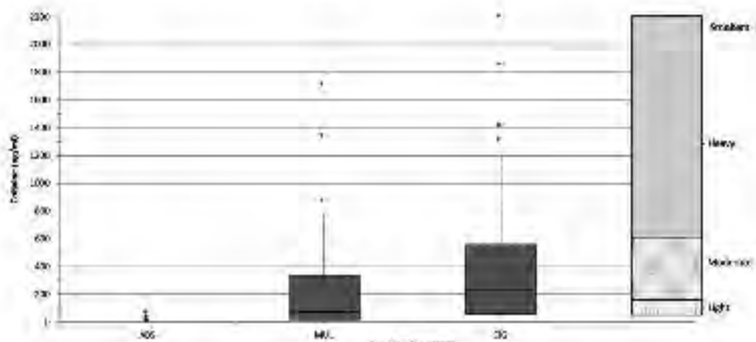

Figure 1. Urinary cotinine level according to consumption groups. Schematic plot consisting of the smallest observation, the lower quartile (bottom of the box), the median (band in the box), the upper quartile (top of the box), and the highest observation still within 1.5 IQR (interquartile range) of the upper quartile. Outliers are presented as asterisk above 1.5 IQR. ABS: abstainers not having used cigarettes or cannabis in the previous 5 days; MUL: cannabis users adding tobacco to the cannabis they smoke but not having smoked cigarettes in the previous 5 days; CIG: cigarette smokers not having smoked cannabis in the previous 5 days.

Table 3. Multivariate Linear Regressions for the Level of Urinary Cotinine

	MUL versus ABS (ref.)		MUL versus CIG (ref.)		MUL versus CIG (≤20) (ref.)		MUL versus CIG (21–60) (ref.)		MUL versus CIG (>60) (ref.)	
Variable	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value
Intercept	−244.7	.369	809.5	.067	425.3	.265	−190.3	.721	1001.5	.173
Consumption profile[a]	197.2	<.001	−78.6	.322	64.7	.301	−200.6	.064	−577.5	.004
Gender (ref.: female)	28.1	.541	77.4	.331	58.4	.348	218.7	.016	83.9	.481
Age	2.3	.797	−4.1	.781	2.2	.857	17.2	.297	−4.1	.858
Ethnicity (ref.: Caucasian)	7.1	.926	−229.1	.111	−36.6	.734	−69.7	.770	−89.8	.763
BMI	9.0	.219	−6.6	.472	−2.2	.762	8.8	.463	−0.7	.960
Passive exposure to tobacco smoke										
At home (ref.: none)	38.2	.460	106.3	.145	39.6	.508	66.6	.421	57.7	.592
At work/school (ref.: never)	−20.7	.643	−34.5	.670	−51.0	.439	−67.5	.424	20.2	.860
In social setting (ref.: less than once)	−37.2	.498	63.2	.666	−152.2	.267	3.6	.951	−1.2	.999
Time since last consumption	—		−7.5	<.001	−4.8	<.001	−6.2	<.001	−5.8	.005

Note. BMI = body mass index.
We provide for each variable the nonstandardized regression coefficient and its associate p value.
[a]Being part of either ABS, CIG, or MUL.

smokers to be lower than in those smoking only cigarettes (Van Vunakis, Tashkin, Simmons, & Clark, 1988). Contrary to our hypothesis, the authors evoked a possible increased metabolism of nicotine originating from enzyme induction through THC to explain their results. To our knowledge, no evaluation of such assumptions has ever been performed.

Works by DiFranza (2009) with youths clearly describe that even a light exposure to tobacco during adolescence confers greater risk to become cigarette smokers on the long run. Hence, significant nicotine exposure through mulling may account for the increased risk of cannabis smokers to initiate cigarettes and, among those who already do, to increase their risk of dependence in adulthood. Along, nicotine exposure through mulling may partly explain a substitution phenomenon observed when adolescents quit or reduce one substance (cannabis or cigarettes) over the other (Akre et al., 2010; Amos et al., 2004). It could also justify the more intense withdrawal symptoms of adolescents quitting both cannabis and cigarettes at the same time (Vandrey, Budney, Hughes, & Liguori, 2008) because amplified nicotine addiction may arise from mulling.

It can be deduced from our results that cannabis users practicing mulling should be considered smokers just like other individuals smoking tobacco products. In countries where mulling is known to be performed, such as the United Kingdom, Canada, Australia, and Switzerland, this should therefore result in the revision of the monitoring criteria for smoking by taking into account the mulling phenomenon. For instance, based on the European School Survey Project on Alcohol and Other Drugs (ESPAD) 2007 data for Switzerland, a nationally representative sample of 7,611 students with a mean age of 15, 24.3% of adolescents smoke cigarettes at least monthly and 3.4% cannabis but no cigarettes (Belanger et al., 2011). If we consider that four out of five young cannabis users in Switzerland do mull frequently, smoking status in the last month would jump to 27.0%, a relative increase of 11%.

Strengths and Limitations

This study has some important strengths. On the one side, biochemical analyses have been blindly performed with the most precise test for nicotine exposure currently available. On the other, statistical analyses have taken into account important factors such as environmental exposure to cigarette smoke. Some limitations must, however, be kept in mind when interpreting our results. First, even if we created a questionnaire comprehensively inquiring the exposure of participants to tobacco and cannabis over a limited period of 5 days, our study is not free of possible recall bias. We also chose not to expose participants to the substances of interest using an observational study design. This may have resulted in factors such as time since the last consumption being of particular importance in multivariate analyses and limited generalizability of our findings. In accordance, mulling behavior from participants in the MUL group may not fully represent that of cannabis users not smoking cigarettes as some may have done so prior to the 5 evaluated days. In addition, the high urinary cotinine level of the MUL group may have been influenced by males being in majority as they are known to have a lower metabolism of nicotine and cotinine than females (Hukkanen et al., 2005). Yet, multivariate analyses took account for such possible confounders. Finally, the minimal sample size of 70 participants per group has not been reached for the MUL group. Nevertheless, significant differences of suitable magnitude have been found despite this statistical limitation.

Implications

Our results indicating that cannabis users are significantly exposed to nicotine through the mulling process have several implications. First, youths seem more and more conscious that cigarettes have adverse effects on health, with some describing cannabis as a more natural alternative (Akre et al., 2010). Yet, cannabis has several adverse health implications (Hall &

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Degenhardt, 2009) and adolescents do add tobacco to the cannabis they smoke. Such incongruity may come from youths not being informed or not considering the amount of tobacco being added to cannabis as significant. They may also consider tobacco as harmful, but only from industrial cigarettes. From a harm-reduction perspective, informing cannabis users about what they expose themselves to through cannabis use could lead them to not initiate, or to stop using cannabis or practicing mulling, resulting in a reduction of their overall substance exposure.

Our study is also of particular interest to health professionals taking care of adolescents and young adults trying to quit cannabis and tobacco products. Professionals should thus be aware of the possible risks of having nicotine withdrawal symptoms resulting from the mulling process. In older adolescents and young adults where a nicotine replacement therapy is planned, clinicians should take into account the nicotine load from mulling. Even if no evidence currently exists on the efficacy of mulling prevention, our study also supports cointerventions on cigarettes and cannabis.

In conclusion, it is important for health professionals supporting quitting or reducing attempts for cigarettes and/or cannabis to ask about the mulling phenomenon and provide information on the subject. If not addressed, nicotine exposure through mulling has all the chances to result in a lower efficacy of any intervention.

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DECLARATION OF INTERESTS

None declared.

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PAPER IN FOREFRONT

Source inference of exogenous gamma-hydroxybutyric acid (GHB) administered to humans by means of carbon isotopic ratio analysis: novel perspectives regarding forensic investigation and intelligence issues

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Abstract γ-Hydroxybutyric acid (GHB) is an endogenous short-chain fatty acid popular as a recreational drug due to sedative and euphoric effects, but also often implicated in drug-facilitated sexual assaults owing to disinhibition and amnesic properties. Whilst discrimination between endogenous and exogenous GHB as required in intoxication cases may be achieved by the determination of the carbon isotope content, such information has not yet been exploited to answer source inference questions of forensic investigation and intelligence interests. However, potential isotopic fractionation effects occurring through the whole metabolism of GHB may be a major concern in this regard. Thus, urine specimens from six healthy male volunteers who ingested prescription GHB sodium salt, marketed as Xyrem®, were analysed by means of gas chromatography/combustion/isotope ratio mass spectrometry to assess this particular topic. A very narrow range of $\delta^{13}C$ values, spreading from −24.81‰ to −25.06‰, was observed, whilst mean $\delta^{13}C$ value of Xyrem® corresponded to −24.99‰. Since urine samples and prescription drug could not be distinguished by means of statistical analysis, carbon isotopic effects and subsequent influence on $\delta^{13}C$ values through GHB metabolism as a whole could be ruled out. Thus, a link between GHB as a raw matrix and found in a biological fluid may be established, bringing relevant information regarding source inference evaluation. Therefore, this study supports a diversified scope of exploitation for stable isotopes characterized in biological matrices from investigations on intoxication cases to drug intelligence programmes.

Keywords Gamma-hydroxybutyric acid · Gamma-butyrolactone · Sodium oxybate · Isotope ratio mass spectrometry · Urine · Source inference · Forensic

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Introduction

γ-Hydroxybutyric acid (GHB) is an endogenous short-chain fatty acid found in mammalian brain tissues as a metabolite of γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter of the central nervous system [1–3]. Biosynthesis also occurs through the peripheral lactonase of γ-butyrolactone (GBL) and the alcohol dehydrogenase of 1,4-butanediol (1,4-BD) into GHB upon direct oral consumption (Fig. 1) [4]. Binding to GHB-specific sites and $GABA_B$ receptors, this molecule exhibits pharmacological properties sought after for specific therapeutic purpose [5, 6]. Indeed, the sodium salt of GHB, referred to as sodium oxybate, is used in the treatment of narcolepsy, with cataplexy, and to help relieve alcohol and opiate withdrawal syndromes [2, 6–8]. Believed to increase the muscle mass due to a stimulatory effect on growth hormone production, GHB became popular amongst body builders as blended to nutritional supplements [9, 10]. Owing to its sedative and

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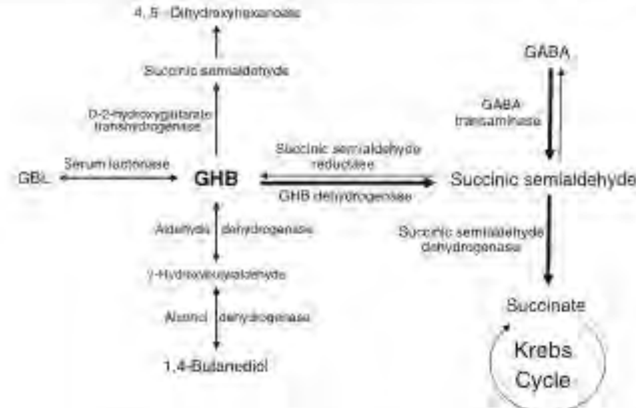
Fig. 1 Simplified metabolic pathway of GHB synthesis and metabolism [8]. The major pathways are shown by *thick arrows*



euphoric effects, this compound quickly gained reputation as a recreational drug due to its acknowledged use at nightclubs and raves [11, 12]. Subsequently, implication of GHB in drug-facilitated sexual assaults emerged, resulting from a combination of heightened sex drive, disinhibition and potential amnesia properties with an ease of spiking in beverages as a colourless and odourless liquid [13]. Whilst trends of both recreative and intoxicating use remain fairly stable, various international studies have reported severe intoxications and overdoses over the last decade, but also significant progression of the GBL consumption phenomenon [2, 14–19].

Therefore, discrimination between endogenous and exogenous GHB in biological fluids, including blood and urine, is often required in toxicological and forensic investigations. However, source identification of this compound is facing a number of challenges. One of the key issues is the short half-life of GHB, with a plasma half-life averaging <1 h, which translates into complete elimination from blood and urine within 6 and 12 h, respectively [20]. When considering the ordinary delay of several hours between drug ingestion and sample collection, along with the endogenous nature of GHB, origin assessment of low urinary or blood concentrations should be carefully interpreted. Accordingly, a general agreement on a cutoff limit for the distinction between endogenous and exogenous GHB is still prone to vast discussions [21–25]. However, this question may be addressed considering alternative biochemical markers. In particular, determination of the carbon isotope composition of this molecule has been proven promising to solve this issue [26, 27]. Indeed, incorporation of ^{13}C to the GHB molecule through biosynthesis appears to differ from synthesis through chemical precursors since the first reflects the C3 and C4 plant diet of an individual, whilst the latest corresponds to its chemical precursors, namely GBL and 1,4-BD, originating from petroleum extracts [28]. Thus, variations in the $^{13}C/^{12}C$ ratio ($\delta^{13}C$ values) allow discriminating between GHB of endogenous and exogenous origins.

Beyond this problem, investigations on intoxication cases and forensic drug intelligence could benefit from such information for the assessment of the linkage between GHB found in a biological sample and drug seizures. Actually, the potential of stable isotope analysis for drug profiling is valuable, as enlightened for cocaine, heroin, amphetamine-based molecules, marijuana and GBL [28–37]. However, this methodology has only been applied to the raw matrix at present, and linking the aforementioned molecules found in biological fluids and in seizure samples remains a promising topic yet to be explored.

Thus, this work proposes to evaluate the likelihood of isotopic fractionation due to metabolism in the body by comparing $\delta^{13}C$ values of prescription pharmaceutical sodium oxybate, marketed as Xyrem®, with urinary GHB of volunteers who ingested this specific prescription drug during a study on sleep. In that respect, measurements were achieved using an extensive sample cleanup procedure followed by the conversion of GHB into GBL prior to analysis by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) [26, 27].

Experimental

Reagents and chemicals

Methanol (≥99.9%), dichloromethane (≥99.9%) and acetic acid (glacial, 100%) were purchased from Merck (Darmstadt, Germany). Acetonitrile (≥99.7%) was obtained from Biosolve B.V. (Chemie Brunschwig, Basel, Switzerland) and hydro-

chloric acid fuming (37%) and sodium chloride (>99.5%) from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). ε-Caprolactone (≥99.0%, Lot no. 087K3521) was supplied by Fluka (Buchs, Switzerland). Ultrapure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a QuantumTM EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA). Helium (Quality 60, >99.9999%) and carbon dioxide gas (quality 40, >99.99%) were obtained from Carbagas (Domdidier, Switzerland). Oasis® MCX cartridge 30 µm (6 cc, 150 mg) were supplied by Waters (Milford, MA, USA) and Bond Elute SAX SPE cartridges 40 µm (3 mL, 500 mg) by Varian Inc. (Palo Alto, CA, USA). GHB enzymatic assay kits were purchased from Bühlmann Laboratories AG (Basel, Switzerland). Sodium oxybate, trademarked as Xyrem® (Lot no. A13870), was obtained from UCB-Pharma SA (Bulle, Switzerland).

GHB urine specimens and prescription medication

Urine specimens were collected from 13 healthy male volunteers, aged 20–26 years (mean age, 23.5±1.6 years), participating in a study on effects on sleep of a sodium oxybate oral preparation, trademarked as Xyrem®. Actually, each subject ingested a sodium oxybate dose equivalent to 30 mg/kg body weight dissolved in 60 mL water twice during the course of this study: once prior to sleep at 11 PM and once prior to a 2-h nap at 3 PM, with a minimum of 1-week interval between both intakes. Urine samples were collected shortly after waking up in the morning and between 9 and 10 PM after the nap, resulting in a 6- to 8-h delay after oral consumption, and stored at −20 °C since collection until GC-MS and GC/C/IRMS analyses. Endogenous levels of GHB were assessed by the collection of urine specimens at six different times over the treatment period and storage under equal conditions. Xyrem® from the batch used for oral administration was analysed as well.

Evidence on in vitro production of GHB in antemortem urine samples has been highlighted as a process that is dependent on storage conditions. Indeed, storage at ambient temperature or refrigerated at 5 °C over a long period of time can result in a significant increase of endogenous GHB concentration [38, 39]. However, keeping urine samples frozen at −20 °C has been reported as significantly reducing this phenomenon to a minimum, even after up to 8 months [40]. Thus, these were the preferred storage conditions for this study.

Quantitative determination of GHB by enzymatic assay

Direct and quantitative determination of GHB in urine has been performed by an enzymatic assay kit on the Dimension® Xpand™ Plus Integrated Chemistry System (Siemens Healthcare Diagnostics SA, Düdingen, Switzerland), an automated chemistry and immunoassay analyser for central laboratories. Reagents, calibrator and validator vials were reconstituted and kept refrigerated at 4 °C prior to analysis. Calibration was performed over the 10- to 100-µg/mL range, with calibration standards at low, medium and high concentration levels (k=3) and validator standards (QC) at two concentration levels (k=2) analysed in triplicate (n=3) each time. A calibration curve was built using a linear regression whose suitability for quantification purpose was verified by comparing concentration measurements of QCs to confidence limits specified in the kit. Eventually, a volume of 12 µL urine was used for each enzymatic assay.

Conversion of prescription pharmaceutical GHB into GBL

In a 10-mL glass tube, 1 mL of 6 M hydrochloric acid was added to 10 µg of Xyrem® prior to vortex mixing for ~30 s. Then, liquid–liquid extraction (LLE) was performed by adding 1 mL dichloromethane and shaking the mixture by inversion for 10 min using a rotator unit. After centrifugation at 2,500 rpm for 5 min, the aqueous layer was transferred and extraction was repeated once with 1 mL dichloromethane. Eventually, the combined organic layer was evaporated in a conical glass tube to ~100 µL under a gentle stream of nitrogen (20 psi) at 25 °C after the addition of 5 µL of 1 mg/mL internal standard (IS) solution (ε-caprolactone in dichloromethane).

Sample preparation

Urine sample cleanup is based on a method previously published and shortened to our particular needs [26]. Considering the concentration of GHB, the volume of urine aliquots was determined as follows:

$$V_{urine} = \frac{10}{GHB_{concentration}} * \mu g, \qquad (1)$$

with V_{urine} in millilitres and $GHB_{concentration}$ in micrograms per millilitre.

Then, urine aliquots were diluted up to a volume of 2 mL with water prior to centrifugation for 5 min at 2,500 rpm. Solid phase extraction (SPE) was performed on an Oasis® MCX cartridge 30 µm (6 cc, 150 mg) previously conditioned by successive addition of 2 mL methanol and 2 mL water. Elution of GHB was carried out with 2.5 mL of methanol/0.1% formic acid in water (10:90, v/v). After evaporation of the eluate to ~0.5 mL under an air stream (20 psi) at 50 °C, further purification was performed on a Bond Elute SAX SPE cartridge 40 µm (3 mL, 500 mg). Conditioning was achieved by successive addition of 2 mL

methanol, 8 mL of 10% acetic acid and 2 mL water at a flow rate of 0.5 mL/min. After loading of the urine extract, interaction with the solid phase occurred for 15 min prior to washing with 1 mL water, 1 mL water/methanol (50:50, *v/v*) and 0.5 mL methanol at a flow rate of 0.5 mL/min. Elution of the analyte was carried out with 3 mL of 10% acetic acid in acetonitrile at a flow rate of 0.5 mL/min. After evaporation of the eluate to dryness under a gentle air stream (5 psi) at 50 °C, the residue was dissolved in 1 mL of 6 M hydrochloric acid prior to vortex mixing for ~30 s. Then, LLE was performed with 1 mL dichloromethane for 10 min using a rotator unit. After centrifugation at 2,500 rpm for 5 min, the aqueous layer was transferred and extraction was repeated once with 1 mL dichloromethane. Eventually, the combined organic layer was evaporated to ~100 µL in a conical glass tube under a gentle stream of nitrogen (20 psi) at 25 °C after the addition of 5 µL of 1 mg/mL IS solution.

GC/C/IRMS analysis

The carbon isotope measurements were performed on a Delta^{Plus} XL IRMS system (ThermoFinnigan MAT, Bremen, Germany) coupled to an Agilent 6890A Gas Chromatograph (HP Analytical Division) via a Finnigan GC Combustion III interface (ThermoFinnigan MAT). The samples were injected using a CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland). The mass spectrometer consisted of an electron impact source held at a 3.0-kV acceleration voltage for CO_2 gas, a magnet and three Faraday collectors for the measurement of the ions at *m/z* 44, 45 and 46. Chromatographic separation was achieved on a DB-17MS capillary column (30 m × 0.25 mm i.d., 0.25-µm film thickness) from J&W Scientific (Folsom, CA, USA). Helium was used as carrier gas with a constant flow of 1.3 mL/min. The GC injection port, combustion oven and reduction oven temperatures were set to 280, 940 and 600 °C, respectively. Standard on–off tests (reference carbon dioxide gas pulses of 20-s duration) were introduced six times during the chromatographic separation. Regarding the analysis of the samples containing GBL and the IS, the oven temperature was increased from 80 °C (5 min) to 240 °C at 20 °C/min, then to 300 °C at 30 °C/min, and maintained at the final temperature for 2 min. The volume of injection was 1 µL and the samples were injected in the splitless mode (1.50 min). Oxidation of the combustion reactor was performed over 1 h after every batch of 20 samples.

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as the parts per thousand deviation of isotopic compositions versus that of Vienna Pee Dee Belemnite and is calculated according to [41].

$$\delta^{13}C\,/‰ = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000 \quad (2)$$

Calibration of the reference gas was previously performed using a mixture of three alkanes (Chiron AS, Trondheim, Norway), C_{15} (*n*-pentadecane), C_{20} (*n*-eicosane) and C_{25} (*n*-pentacosane), with $\delta^{13}C$ values of −30.22‰, −33.06‰ and −28.21‰, respectively.

Acquisition and evaluation of the GC/C/IRMS data were performed with the ISODAT 2.5 software (ThermoFisher Scientific, Bremen, Germany).

GC-MS analysis

Prior to GC/C/IRMS analysis, identification of the substance was ensured by GC-MS chromatographic retention time and by measurement of the full EI-MS spectrum between *m/z* 40 and 300. The diagnostic ions selected for the identification of each compound were the following: GBL (*m/z* 56, 86 and 42) and ε-caprolactone (*m/z* 55, 75, 84 and 114).

GC-MS analysis was performed on a Hewlett-Packard 5890 Serie II Plus chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7673 autosampler and coupled with a HP 5971 mass selective detector. GC separation was achieved on a DB-17MS capillary column (30 m×0.25 mm i.d., 0.25-µm film thickness) from J&W Scientific. Helium was used as carrier gas with a constant flow of 0.8 mL/min and at the initial column head pressure of 15 psi. For a robust identification of the target compounds, the GC operating conditions were identical to GC/C/IRMS analysis.

Identification criteria

Identification criteria were defined according to a technical document addressing this particular topic [42]. The chromatographic retention time (t_R) tolerance window must be within ±1% of the retention time of the reference material analysed in the same batch. Concerning full EI-MS experiments, at least two diagnostic ions are required, with the relative intensity of any of the ions not differing by more than 20% from that of the quality control material. A signal-to-noise ratio >3 must also be considered.

Data treatment and analysis

The $\delta^{13}C$ values were analysed statistically using S-PLUS® 7.0 for Windows. For distribution testing, a Kolmogorov–Smirnov test of normality has been employed. Equality of the

variances was assessed using a Levene test. Then, statistical differences among samples were tested using the two-sample t test, with $p<0.05$ considered statistically significant.

Results and discussion

Quantitative determination of GHB in urine by enzymatic assay

Quantification by enzymatic assay has been favoured for the reason that virtually no sample cleanup was required, providing a high throughput particularly suitable for use as a screening procedure. Indeed, such method allowed targeting samples of interest in a rapid and straightforward fashion. Noteworthy is that endogenous levels of GHB in urine specimens collected at six different times over the treatment period and reliability of storage conditions could be assessed simultaneously along with this single batch of analyses. Also, storage condition effects were limited as this procedure allowed avoiding an additional freeze and thaw cycle before urine extraction for compound identification and isotopic measurements. Actually, this simple and fast quantification method could be followed by a sample preparation for GC/MS and GC/C/IRMS analyses within ~1 h after thawing of the urine specimens.

A concentration range comprising expected urinary levels of both endogenous and exogenous GHB was initially determined, with consideration of the performance characteristics described in technical notes supplied with the enzymatic assay kit. Thus, calibration was performed over the 10- to 100-μg/mL range using calibrators at the 10-, 50- and 100-μg/mL concentration levels (k=3) analysed in triplicate (n=3). Due to the linear response, unweighted linear least-squares regression was chosen for quantification purpose, with R^2 corresponding to the calibration curve being >0.99. Also, the suitability of direct quantification of GHB in urine over the assay range was established as the concentration values obtained for calibration standards and standard deviations (SD) met the guidelines specified in the aforementioned technical notes. Also, samples exceeding the upper limit of quantification were diluted 1:10 (v/v) with 0.9% sodium chloride solution, as indicated by the manufacturer, and concentration values were multiplied by 10.

Following quantification, urine specimens of interest for further extraction and isotopic measurement were reduced down to nine samples originating from seven subjects. Indeed, in order to avoid more extensive purification steps prior to IRMS measurements and to support the exogenous origin of GHB found in urine, only samples with a concentration above 20 μg/mL were selected (Table 1). Noteworthy is that a few volunteers presented levels of GHB lower than 10 μg/mL even after treatment with Xyrem®, which may be explained by the short half-life of this substance joint to a longer delay before urine collection due to a prolonged sleep. Additionally, endogenous GHB in urine specimens collected over the treatment period was not detected. This ensured that subjects involved in this study did not suffer from 4-hydroxybutyric aciduria, which would result in naturally elevated endogenous levels of GHB [43, 44], and also excluded potential in vitro production issues.

Identification of GBL by GC/MS analysis

GC/MS analysis of Xyrem® and urine specimens allowed the identification of target compounds and suitability assessment for subsequent isotopic measurements. Satisfactory sample cleanup was achieved for eight of the nine samples, corresponding to six of the seven volunteers. Indeed, identification criteria were met for GBL and ε-caprolactone, with adequate chromatographic resolution and complete conversion of GHB into GBL (Fig. 2), except for one sample due to a co-eluting compound. Whilst the nature of that molecule could not be clearly identified, an exogenous origin may be hypothesized as there was no previous observation of this compound in urine extracts, even in trace amount. Thus, the latter was not submitted to

Table 1 Quantification of GHB by enzymatic assay and carbon isotopes determination in urine specimens of interest by GC/C/IRMS (n=8)

Type	Subject	Concentration (μg/mL)	$\delta^{13}C$ (‰)	SD (‰)
Urine specimen	1	86.9	−24.97	0.06
		76.5	−24.81	0.25
	2	168.6	−24.92	0.08
		97.4	−24.92	0.13
	3	33.5	−25.02	0.20
	4	22.6	−25.05	0.12
	5	39.6	−25.06	0.25
	6	35.5	−24.89	0.25
Standard	Xyrem®		−24.99	0.02

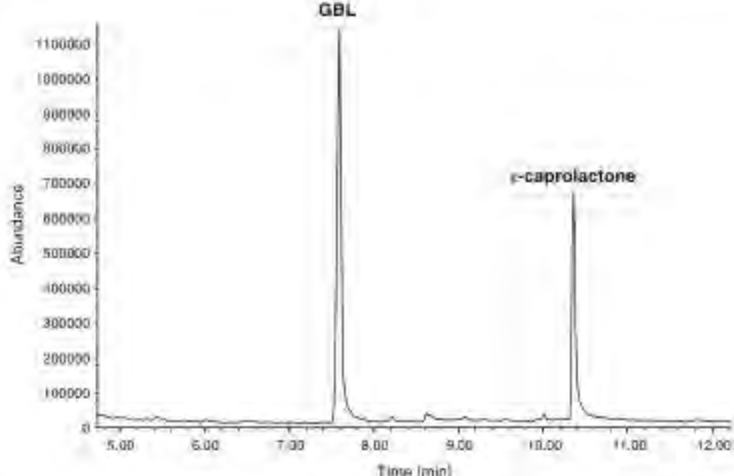


Fig. 2 GC-MS chromatogram of a urine specimen containing GHB at a concentration of 100 µg/mL and ε-caprolactone at 50 µg/mL

IRMS analysis. Also, Xyrem® was found to be of excellent purity.

Isotope measurements of GBL in urine by GC/C/IRMS

Conversion of GHB into GBL was favoured over conventional derivatization with di-TMS in order to avoid the addition of carbon atoms to the molecule [26, 45]. Indeed, subsequent calculation of a correction factor accounting for this phenomenon is necessary, resulting in $\delta^{13}C$ values with a larger SD [27]. Alternatively, conversion into GBL leads to the loss of a molecule of water, with no influence on carbon atoms attached to the original molecule of GHB. Thus, translation of isotopic measurements of GBL into $\delta^{13}C$ values for GHB is straightforward.

Each sample preparation was spiked with ε-caprolactone, a molecule very close in structure to GBL and displaying a slightly different chromatographic retention. Potential mass discrimination during the course of GC/C/IRMS analysis was tested with ε-caprolactone serving as internal standard [28]. The reproducibility of isotopic measurements was assessed accordingly, relying on the $\delta^{13}C$ value of the IS calibrated previously to this study (mean $\delta^{13}C$ value=−23.40‰, SD=0.21‰, n=30), using a 95% confidence interval as a run acceptance criteria. Also, the stability of the system was evaluated introducing reference carbon dioxide gas pulses (20-s width) six times during the chromatographic separation, and pulses at 420 and 690 s were used to normalize $\delta^{13}C$ values (Fig. 3).

Prior to the isotopic measurements of urine specimens, the linear response of the IRMS was defined by injecting different amounts of GHB converted into GBL from Xyrem®. Indeed, the accuracy of the carbon isotopic ratio determination may be significantly affected when the signal intensity is outside the linearity range [46]. Thus, increasing quantities of GBL from 10 to 100 ng were injected, resulting in signal intensities ranging from 389 to 4,432 mV, respectively. A linear response was observed, as demonstrated by the stable $\delta^{13}C$ values (0.03‰ per millivolt) obtained.

Each urine sample was extracted in triplicate, and the isotopic measurements of GHB are summarized in Table 1. The $\delta^{13}C$ values determined in this study range from −24.81‰ to −25.06‰, with relatively small deviations (SD<0.26‰). The stability of IRMS measurements during the chromatographic separation proved satisfying, as verified by the standard on–off tests (mean $\delta^{13}C$ value=−28.50‰, SD=0.10‰, n=162 for six reference carbon dioxide gas pulses). Likewise, reproducible $\delta^{13}C$ values were collected for the IS (mean $\delta^{13}C$ value=−23.38‰, SD=0.19‰, n=27), with every carbon isotope ratio comprised in the confidence interval previously established (two-sample t test).

Noteworthy is that the distribution of $\delta^{13}C$ values did not show significant deviation from the isotopic values and related SD of Xyrem® (mean $\delta^{13}C$ value −24.99‰, SD=0.02‰), as revealed by statistical tests. Indeed, equality of the variances was verified using a Levene test and statistical differences among urine specimens were ruled out by the two-sample t test, with $p<0.05$ considered statistically significant. Thus, metabolism of GHB did not induce a significant carbon isotopic fractionation, as reported for each of the six subjects.

Several pharmacokinetic studies described the conversion of GHB to succinic semialdehyde and further to succinate prior to entering into the Krebs cycle as the major

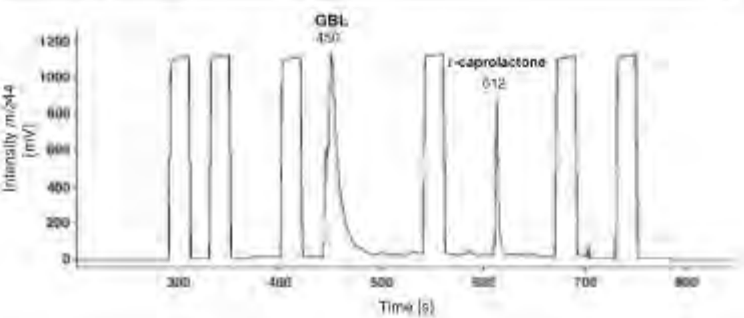


Fig. 3 GC/C/IRMS chromatogram (m/z 44) of a urine specimen containing GHB at a concentration of 100 μg/mL and ε-caprolactone at 50 μg/mL. The *square-topped peaks* represent pulses of CO_2 reference gas

metabolic pathway, along with other minor metabolic routes (Fig. 1) [8, 47, 48]. Due to this extensive hepatic metabolism, <1% of the dose is excreted unchanged in urine. Thus, verifying whether the carbon isotopic composition of this small amount of GHB recovered in urine displayed variations with respect to the original pharmaceutical preparation was a crucial point. Indeed, reduction of GHB into succinic semialdehyde by GHB dehydrogenase may lead to carbon isotopic fractionation due to the kinetic isotope effect. Considering such effect, the reaction rate of ^{13}C-enriched isotopologues is known to be slower in bond making or breaking chemical processes [49, 50]. Therefore, the small fraction of GHB found unchanged in urine may potentially exhibit ^{13}C enrichment compared to the isotopic signature of the original dose administered to the subjects. Also, renal reabsorption and metabolic clearance of GHB could be hypothesized as an additional source of carbon isotopic fractionation. However, determination of the carbon isotope ratio for each of the six subjects did not highlight variations in the isotopic composition through GHB metabolism as a whole. Accordingly, the potential sources of carbon isotopic fractionation previously mentioned should not significantly affect the $\delta^{13}C$ values.

Our findings tend to demonstrate the possibility of connecting GHB in biological samples and this substance as a prescription or illicit drug by means of carbon isotope determination. Noteworthy is that Xyrem® quantity administered to the volunteers corresponded to an average dose for recreational use, which is significantly less than both therapeutic and intoxicating doses. Therefore, the methodology applied in this study could be used at ease with higher urinary concentrations of GHB. Such findings would be very valuable when investigating intoxication cases and in support of drug intelligence as they may fill the gap between the raw matrix and biological fluids with valuable information. Indeed, corresponding carbon isotope contents would allow linking drug seizures to urine specimens, and by extension drug traffickers to individuals charged with substance abuse felony or suspicious individuals to drug poisoning victims. This would bring an additional level of evidence to tackle the aforementioned issues of forensic interest. In addition, considering the increasing popularity of GBL consumption, such studies could be followed up by the assessment of metabolism influence on the carbon isotopic profile of GBL excreted as GHB in biological fluids.

Conclusion

Determination of the carbon isotope content of GHB by GC/C/IRMS, performed with a previously published method [26], was applied to eight urine specimens of six healthy male volunteers who ingested pharmaceutical GHB sodium salt, known as sodium oxybate and trademarked as Xyrem®, as part of a study on sleep. A very limited range of $\delta^{13}C$ values, from −24.81‰ to −25.06‰, was observed, corresponding to the carbon isotopic values of Xyrem® (mean $\delta^{13}C$ value = −24.99‰) used for treatment. Since urine samples and prescription drug could not be discriminated by means of statistical analysis, metabolism of GHB demonstrated no significant influence on $\delta^{13}C$ values.

This study provides a baseline for further studies and for the exploitation of stable isotopes characterized in biological matrices in both intoxication cases brought to court and drug intelligence programmes. Indeed, source inference subsequent to carbon isotope determination appears achievable as highlighted by the link established between GHB as a raw matrix or found in a biological fluid. In addition, such studies may be extended to GBL, a chemical precursor of increasing interest amongst substance users, to assess potential isotopic fractionation related to its metabolism prior to excretion as GHB in biological fluids.

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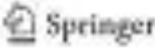

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Potential of IRMS technology for tracing gamma-butyrolactone (GBL)

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ABSTRACT

Popularity of γ-hydroxybutyric acid (GHB) is fairly stable among drug users, while the consumption of its chemical precursor, γ-butyrolactone (GBL), is a growing phenomenon. Although conventional analytical methods allow to detect this substance in various matrices, linking a trace and a source is still a difficult challenge. However, as several synthesis pathways and chemical precursors exist for the production of GBL, its carbon isotopic signature may vary extensively. For that purpose, a method has been developed to determine the carbon isotopes content of GBL by means of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS).

The $\delta^{13}C$ values of 19 bulk samples purchased worldwide were in the range from −23.1 to −45.8‰ (SD < 0.3‰). Furthermore, testing on the purification of GBL by distillation has not been found to be consistent with such a large range of $\delta^{13}C$-values, which are likely to result from the isotopic composition of the organic precursors used to produce GBL together with the kinetic isotope effect associated with the synthesis routes. Finally, inter- and intra-variability measurements of the $\delta^{13}C$-values demonstrated the high potential of IRMS for discriminating between seizures of GBL and for source determination.

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1. Introduction

The demand for γ-butyrolactone (GBL) has increased in the last decade, mainly as a chemical intermediate to manufacture polymers, but also as a biodegradable degreaser or paint remover [1]. In 2002, the domestic production in the USA was estimated at 80,000 tons per year [2]. The total capacity of GBL manufacturing in China was reported in 2006 to be around 50,000 tons per year [3]. As illustrated in Fig. 1, several chemical intermediates may be potentially converted into GBL at the industrial level. The major portion of GBL is currently being produced via the dehydrogenation of 1,4-butanediol (1,4-BD) [4–6], which is manufactured from the reaction of acetylene with formaldehyde; this reaction is known as the Reppe process [7]. New manufacturing routes of GBL are based on the two-stage hydrogenation of economically attractive raw materials such as dimethyl maleate [8] or maleic anhydride [9–11]. Tetrahydrofurane can also be used as a precursor to synthesize GBL following a single oxidation step [12].

Recreative use of γ-hydroxybutyric acid (GHB) and to a lesser extent GHB-facilitated sexual assaults ('date rape') is a relatively recent and stable phenomenon among European countries [13,14]. However, recent surveys indicate that consumption of its chemical precursors, namely GBL and 1,4-BD, is a growing trend among drug users due to several promoting factors [15]. Starting with these materials, the synthesis of GHB is rather simple and, most remarkably, these precursors exhibit a rapid conversion into GHB by peripheral lactonase upon direct oral consumption [2,16–18]. Moreover, both GBL and 1,4-BD would hardly be regulated under a national or an international legislation as they are important and common industrial solvents used in large quantities in the synthesis of plastics and polymers [13,19]. The lack of control coupled with the availability of these substances on the internet for a relatively cheap price increase the popularity of GHB, GBL and 1,4-BD consumption. Therefore, there is a need to develop analytical means to assist law enforcement agencies disrupt the use and trafficking of GHB and its precursors.

In forensic cases, the presence of GHB or precursors is investigated in items seized at the premises, in the form of drug samples or spiked beverages, but also as biological samples (urine or blood) collected from drug users or sexual assault victims. Although these substances may be detected by conventional analytical methods, any linkage between trace and source is difficult to ascertain. However, as these substances can be synthesized through many different routes using a diversity of chemicals, variations in their stable isotopes content may be potentially observed. In that respect, several investigations of

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Fig. 1. Structures and synthesis pathways of synthetic precursors of γ-butyrolactone.

stable isotopes have been conducted to assist in determining the manufacturing source of illicit synthetic drugs such as amphetamine type stimulants based molecules [20].

In the present study, an appropriate and robust gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) method has been developed and tested for the characterization of GBL samples. Then, the variability in the carbon isotopic compositions between samples of GBL purchased from different chemical providers and internet retailers of various countries in the world has been studied. The distillation effect has also been evaluated as it takes place in the production of GBL and thus may possibly affect the isotope ratio value. Finally, the discriminating ability of IRMS to infer the source of GBL samples will be discussed. This work provides a baseline for future studies and some explanation to understand the variations in the carbon isotope composition of GBL.

2. Materials and methods

2.1. Chemicals

Dichloromethane (≥99.9%) was purchased from Merck (Darmstadt, Germany). γ-Butyrolactone (GBL) was purchased from Fluka (≥99.0%, Lot 1081369770, Buchs, Switzerland), Sigma (≥99.0%, Lot 087K3523, Buchs, Switzerland), Lipomed (≥99.0%, Lot 823.1B0.1L5, Arlesheim, Switzerland) and Wako (≥99.0%, Lot PEN0970, Osaka, Japan). ε-Caprolactone (≥99.0%, Lot 1250826) was obtained from Fluka (Buchs, Switzerland). Helium (Quality 60, ≥99.9999%) and carbon dioxide gas (Quality 40, ≥99.99%) were purchased from Carbagas (Domdidier, Switzerland). Tetradecanoic acid methyl ester (C14:0, #14M, $C_{15}H_{30}O_2$, $\delta^{13}C$ = −29.98 ± 0.02‰, ≥99.0%) was obtained from Arndt Schimmelmann (Indiana University, Department of Geological Sciences, Biogeochemical Laboratories, 1001 East 10th Street, Bloomington IN USA).

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Bulk GBL was purchased from different internet retailers: Alloycleaner (#1, London, United Kingdom), Cleanpower (#2, Bytom, Poland), Cleanstar24 (#3, Tarnowo Dolna, Poland), Everchem (#4, China), GBL24 (#5, Bialystok, Poland), GBL Cleaner (#6, Uden, Netherlands), Liquidsoap (#7, Coco Islands, United Kingdom), Multicleaner (#8, Nijmegen, Netherlands), Shine&Bright (#9, Nijmegen, Netherlands), Eastar Chem Industrial Corp. (#10, China), Nanjing KaiKai Technology Co. Ltd. (#11, China), Taizhou Synhwa PharmaChem Co. Ltd. (#12, China) and two different batches from Anhui Huaxing Chemical Industry Co. Ltd. (#13 and 14, China) ordered at 6 months interval.

2.2. Sample preparation

In a 10 mL glass tube, 150 μL of ε-caprolactone (Internal Standard, 20 μg/mL in dichloromethane) and 50 μL of tetradecanoic acid methyl ester (Isotope Calibrator, 10 μg/mL in dichloromethane) were added to 100 μL of GBL (100 μg/mL in dichloromethane). After vortex-mixing for 5 s, the solution was transferred to an auto-sampler vial with a 300 μL insert for both GC–MS and GC/C/IRMS analyses.

2.3. Description of the distillation apparatus

A standard distillation design was employed for the study of the carbon isotope fractionation of GBL during evaporation. An aliquot of 100 mL GBL was introduced into a 250 mL two-neck round-bottom flask connected to a distillation head. A thermometer was connected to the two-neck round-bottom flask above bulk GBL to record the temperature of the escaping vapor. The distillation head was composed of a 15-cm long neck at the top of which a standard thermometer was fixed to follow the temperature of the vapor during distillation and a lateral condenser whose inner heat exchange tube is 25 cm long. The condenser was cooled with water at ambient temperature (~23 °C). Four 50 mL round-bottom flasks were connected to the condenser using a distillation receiver. A heating mantle was used to slowly heat the bulk sample to a temperature of 204 °C, corresponding to the boiling point of GBL. Complete distillation was performed within 2 h and allowed to collect four distillate fractions of 25 mL each. For comparison purposes three distillations were performed for each bulk GBL and each distillate fraction was measured in triplicate by means of GC/C/IRMS.

2.4. GC/C/IRMS analysis

The carbon isotope measurements were performed on a Delta V Plus IRMS system (ThermoFisher Scientific, Bremen, Germany) coupled to a TraceGC Ultra Gas Chromatograph via a GC-C/TC III interface (ThermoFisher Scientific, Bremen, Germany). The samples were injected via a TriPlus™ autosampler (ThermoFisher Scientific, Bremen, Germany). The mass spectrometer consisted of an electron impact source held at 3.0 kV acceleration voltage for CO_2 gas, a magnet and three Faraday collectors for measurement of the ions at m/z 44, 45 and 46.

Chromatographic separations were achieved on a DB-17MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA). Helium was used as carrier gas with a constant flow of 1.2 mL/min. The GC injection port, combustion oven and reduction oven temperatures were set to 280 °C, 940 °C and 600 °C, respectively. Reference carbon dioxide gas pulses (20 s durations) were introduced at five different times during the course of the chromatographic separation. Regarding the analysis of the samples containing GBL, the internal standard (IS) and the internal calibrator (IC), the oven temperature was increased from 50 °C (3 min) to 150 °C at 20 °C/min, then to 300 °C at 30 °C/min, and maintained at the final temperature for 3 min. The volume of injection was 1 μL and the samples were injected in the splitless mode (1.50 min). Oxidation of the combustion reactor was performed over 1 h after every batch of 30 samples.

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Vienna Pee Dee Belemnite (VPDB) and is calculated according to [21]:

$$\delta^{13}C_{(‰)} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000 \quad (1)$$

Acquisition and evaluation of the GC/C/IRMS data were performed with the ISODAT 2.5 software (ThermoFisher Scientific, Bremen, Germany).

2.5. GC/MS analysis

Prior to GC/C/IRMS analysis, identification of the substance was ensured by GC chromatographic retention time in agreement within 1% of the retention time of reference material analysed in the same batch and by measurement of full EI MS spectrum between m/z 40 and 300 with an acceptable maximum tolerance edited in a technical document [22]. The diagnostic ions selected for identification of each compound were the following: GBL (m/z 56 and 86), ε-caprolactone (m/z 55, 75, 84 and 114) and tetradecanoic acid methyl ester (m/z 55, 74, 87, 143, 199 and 242).

The GC/MS analyses were performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph (HP Analytical Division, Waldbronn, Germany) equipped with a HP 7673 auto-sampler and coupled with a HP 5971 mass selective detector (MSD). GC separation was achieved on a DB-17MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA). Helium was used as carrier gas with a constant flow of 0.8 mL/min and at the initial column head pressure of 15 psi. For a robust identification of the target compounds, the GC operating conditions were identical for both GC–MS and GC/C/IRMS analyses. The oven temperature was increased from 50 °C (3 min) to 150 °C at 20 °C/min, then to 300 °C at 30 °C/min and maintained at the final temperature for 3 min. Injections of samples (1 μL) were made at 280 °C in the splitless mode. EI mass spectra were recorded with the instrument tuned by continuous scanning in the 40–300 amu range at an ionization potential of 70 eV.

2.6. Data treatment and analysis

The $\delta^{13}C$-values were analysed statistically using S-PLUS® 7.0 for Windows. For distribution testing, a Kolmogorov–Smirnov test of normality has been employed. Equality of the variances was assessed using a Levene test. Then statistical differences among groups were tested using the two-sample t-test with $p < 0.05$ considered statistically significant.

The evaluation of the overlapping zone between the inter-variability and the intra-variability of the distribution $\delta^{13}C$-values was performed by studying the behaviour of the ROC (receiver operating characteristic) curves [23].

3. Results and discussion

From the early 1990s, extended research has been conducted on drug profiling in the area of analytical method development and statistical data treatment. Methodologies have been established for the comparison of illicit drug seizures in an intelligence-led perspective using large databases [24]. These methods mainly use GC/MS for the analysis of samples in order to obtain intelligence information.

Basically, some drugs are produced through a number of different routes. Therefore, the organic impurities profile which will be influenced by the precursors and chemicals can provide valuable information to assess the link between drug seizures, production batches and trafficking routes [25–27]. Regarding profiling of GBL, it appears that this approach will not be effective as this chemical is manufactured at high purity level (>99%). GC–MS analysis of the samples purchased from different internet retailers and obtained from police seizures confirmed indeed the presence of impurities at trace level. Alternatively, the determination of differences in the carbon isotope ratio of GBL by means of IRMS was found to be a more promising methodology to tackle the links between GBL samples. Valuable use of stable isotope profiles has already been assessed in order to gain intelligence through forensic analysis, for instance for the determination of the geographic origin of drugs [28], or for linking samples of drugs [29] or black powder [30].

Based on previous work, GBL may be analysed by means of GC/C/IRMS using a moderately polar column and hence does not require a chemical modification to provide acceptable chromatographic characteristics [31]. In that study, it is worth to note that each sample preparation was spiked with ε-caprolactone and tetradecanoic acid methyl ester. Tetradecanoic acid methyl ester was added as a standard of known isotopic composition to calibrate the target compounds, while ε-caprolactone, a lactone displaying slightly different chromatographic retention than GBL, was used as an internal standard compound to test for potential mass discrimination during the course of GC/C/IRMS analyses [32]. An example of a GC/C/IRMS chromatogram of GBL, ε-caprolactone and tetradecanoic acid methyl ester is shown in Fig. 2. Reference carbon dioxide gas pulses (20 s width) were introduced five times during the chromatographic runs to check for IRMS stability.

Prior to the determination of the carbon isotopic ratio of the lots of GBL, analysis of different quantities of GBL from the same batch was performed to define the linear response of the IRMS. This parameter is crucial as it might be that accuracy of GC/C/IRMS is significantly affected when signal intensity is outside the linearity range [33]. Typically, it resulted in stable $\delta^{13}C$-values (0.09‰/V) for

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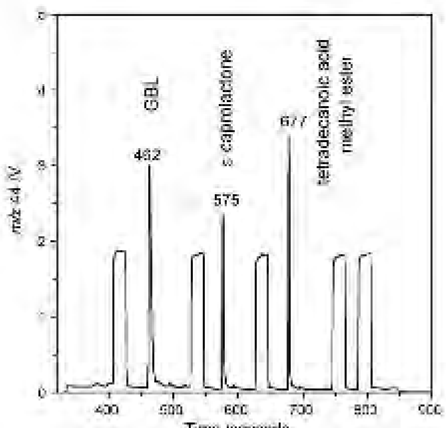

Fig. 2. GC/C/IRMS chromatogram (m/z 44) of GBL, ε-caprolactone and tetradecanoic acid methyl ester. The square-topped peaks represent pulses of CO_2 reference gas.

the signal intensities between 0.5 and 4 V on the m/z 44 channel corresponding to injected amounts of 1 and 10 ng, respectively.

Table 1 lists the carbon isotopic data of GBL samples obtained from chemical providers and internet retailers (GBL#1–14). The δ^{13}C-values of these GBL samples ranged from −23.1 to −45.8‰, and were associated with standard deviations lower than 0.3‰ of triplicate analysis. The stability of IRMS measurement during the chromatographic separation was verified by the standard-on-off tests (SD < 0.21‰ for 5 reference CO_2 pulses). Furthermore, reproducible δ^{13}C-values for ε-caprolactone (IS) were obtained (mean δ^{13}C-value = −26.15‰, SD = 0.14‰, n = 114), thereby enabling a 95% confidence interval as a run acceptance criteria. Noteworthy, distribution of the δ^{13}C-values of the internal standard showed no significant deviation from Gaussianity ($p > 0.05$) as revealed by the Kolmogorov–Smirnov test. The GBL samples were analysed again in triplicates 6 months later and did not show significant deviations from the mean δ^{13}C-values and related SD listed in Table 1. Finally, all δ^{13}C-values of the IS were comprised in the confidence interval established previously (two-sample t-test). The repeatability of the ^{13}C measurements is of major importance in a forensic context in order to create and maintain a carbon isotope ratio database of GBL samples.

A relatively large range of δ^{13}C-values has been determined in that study for the GBL samples purchased from various internet retailers and chemical providers. A variation of −23‰ in the δ^{13}C-values of GBL is equivalent to a difference in the ^{13}C isotope abundance of 0.025 at.%. Scanning MS in selected ion monitoring (SIM) might only achieve reliable analyses of isotopic composition at natural abundance level of 0.1 at.% [34,35]. Hence, it would not be conceivable for our purpose to perform carbon isotope analyses using a conventional mass spectrometer.

GBL production may include a purification step, generally a distillation, to obtain the product in a pure form [9]. Therefore, a possible isotope fractionation due to the thermodynamic isotope effect should also be considered [20]. Distillation of solvent such as methanol, chloroform and benzene were found to lead to a slight depletion of ^{13}C between the original vapor phase collected and consecutive fractions [36]. A comprehensive explanation of these results at the molecular level was reported subsequently [37]. To assess the magnitude of isotopes fractionation by distillation of GBL, IRMS analysis of GBL collected over consecutive distillates fractions was performed. For instance, distillations of 100 mL bulk GBL#11 resulted in a slight depletion of ^{13}C between consecutive 25-mL fractions (−27.7‰ for the first distillate versus −28.5‰ for the last one). Based on these results, purification by means of distillation will not affect the δ^{13}C-values dramatically, at least it may not provide an explanation for the wide range of δ^{13}C-values reported in this study.

On the basis of the current knowledge, it can be assumed that the broad range of δ^{13}C-values for GBL is probably the consequence of the variability in the carbon isotope composition of the organic precursors which will be conserved through to the product GBL. To test this hypothesis, batch-to-batch variations were assessed for bulk GBL (GBL#13–14) obtained from an industry using the Reppe process to manufacture 1,4-BD. As main outcome, statistical analysis did not reveal any evidence against the assumption that the mean δ^{13}C-values were equal. Hence, it is likely that the δ^{13}C-values of GBL were inherited in that case from those of acetylene and formaldehyde, two precursors which served to synthesize 1,4-BD in a first

Table 1
δ^{13}C-values of GBL samples obtained from chemical providers and internet retailers (GBL#1–14). Both series of analyses were performed at 6 months interval.

Type	Sample id	Country of purchase	1st series of analyses		2nd series of analyses	
			δ^{13}C-value/‰	SD/‰	δ^{13}C-value/‰	SD/‰
Standard	Fluka	Switzerland	−45.1	0.06	−44.9	0.14
	Lipomed	Switzerland	−31.7	0.12	−31.8	0.07
	Riedel de Häen	Germany	−36.4	0.12	−36.3	0.10
	Sigma	Switzerland	−35.4	0.08	−35.4	0.12
	Wako	Japan	−26.0	0.10	−26.0	0.12
Internet retailers	#1	UK	−24.3	0.09	−24.2	0.07
	#2	Poland	−45.5	0.02	−45.5	0.06
	#3	Poland	−45.8	0.05	−45.8	0.14
	#4	China	−30.6	0.05	−30.6	0.12
	#5	Poland	−45.5	0.18	−45.5	0.05
	#6	Switzerland	−32.1	0.11	−32.1	0.06
	#7	UK	−27.9	0.11	−28.0	0.09
	#8	Netherlands	−29.8	0.15	−29.7	0.13
	#9	Switzerland	−23.1	0.02	−23.2	0.06
	#10	China	−29.5	0.05	−29.4	0.01
	#11	China	−27.9	0.13	−28.1	0.07
	#12	China	−29.8	0.05	−29.8	0.09
	#13	China	−27.8	0.05	−27.9	0.05
	#14	China	−27.6	0.09	−27.7	0.08

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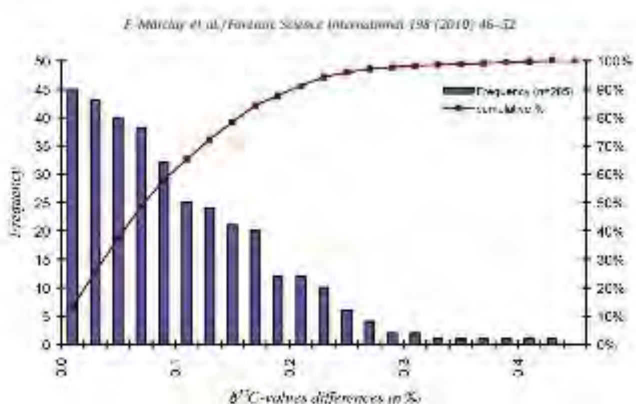

Fig. 3. Intravariability: distribution of differences in $\delta^{13}C$-values of GBL between pairs of samples from the same batch.

step. Given the relatively low cost for producing GBL at the industrial level, it may be expected that the organic precursors are mainly originating from petroleum and related organic matter. It was reported by Yeh and Epstein [38] that the $\delta^{13}C$-values of 114 petroleum samples were ranging from −23 to −33‰ depending on their geographic age or location; more importantly, the study pointed out that compound-grouped fractions did not show significant isotope fractionation with respect to the crude oil.

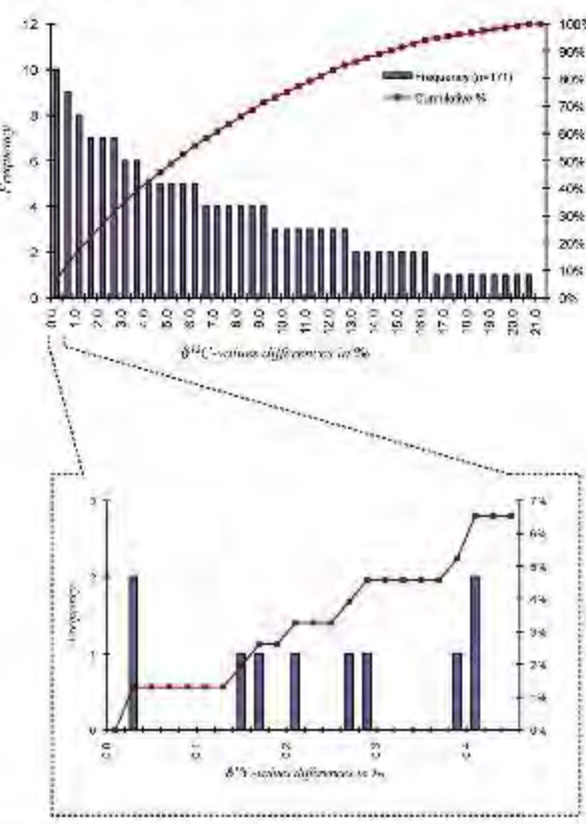

Fig. 4. Intervariability: distribution of differences in $\delta^{13}C$-values of GBL between pairs of samples from different batches. The second plot (below) shows the same distribution for differences in $\delta^{13}C$-values between 0.0 and 0.4‰; this corresponds to the range of the distribution of intra-variability illustrated in Fig. 3.

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Considering that the isotopic signature of crude oil feedstocks is conserved throughout the chemical process, these data may not account for $\delta^{13}C$-values less than −40‰ assessed for some of the GBL samples (Table 3). Likewise, n-butane used as a feedstock for the manufacture of maleic anhydride is not likely to induce large variation in the carbon isotopic compositions of bulk GBL [39]. In contrast, the kinetic isotope effect on the reactions to produce GBL may contribute significantly to the isotope distribution observed herein. Non-quantitative organic reactions characterized by uncomplete conversion of the reactant containing the carbon bond involved in the rate determining step is susceptible to induce an isotope fractionation at that specific carbon position [40]. Therefore, it may be hypothesized that an isotope fractionation occurred during the manufacturing process of GBL samples (for instance GBL#2, GBL#3 and GBL#5), owing to the unusual ^{13}C depletion (∼−45‰) observed for these samples.

Potential of IRMS to infer the source of GBL seizures has been evaluated in a next step. This has been done by assessing the inter- and intra-variabilities of the $\delta^{13}C$-values. The intra-variability was calculated by reporting the differences in the carbon isotope ratio between each of the six replicates of the 19 GBL bulk (285 values). Concerning the inter-variability, the differences in the carbon isotope ratio means between the 19 GBL standards (171 values) have been measured. The distributions of the intra- and inter-variabilities were compared using a visual plot (Figs. 3 and 4) and a ROC curve (Receiver Operating Characteristic) leading to a high discrimination between the two populations (area under the curve of 0.991).

These results, associated with an accuracy of less than 0.3‰ and a high repeatability, suggest that the discriminating ability of IRMS is sufficiently high to assess the source of a GBL sample. In order to test this approach, future work will be focused on the analyses of GBL samples from police seizures.

4. Conclusions

A method for the determination of the carbon isotopes content of GBL by GC/C/IRMS was developed and its accuracy and robustness were assessed. Hence, this method has been applied at 6 months interval to 19 GBL bulk lots purchased from diverse chemical providers and internet retailers of different countries, showing a high repeatability. The wide range of $\delta^{13}C$-values (from −23.12 to −45.78‰) enlightened the broad variability in stable isotope profiles that characterizes GBL batches from different sources and origins. In accordance with previously published research conducted with several solvents, it has been verified that distillation which could take place in the purification process of GBL production does not involve isotope fractionation and thus does not significantly affect $\delta^{13}C$-values.

The results obtained so far demonstrate the feasibility of a forensic approach to discriminate between samples of different origins by means of statistical analysis and further link a specimen to a definite source or draw inference on a possible common source of two samples. Considering the measurements performed, variations in the $^{13}C/^{12}C$ ratio of GBL are likely to result from the combination of the stable isotope profile of the chemicals used in the manufacturing processes and the broad diversity of the synthesis pathways. In order to study further the influence of these parameters, isotopic profiling of GBL samples manufactured in a same industrial plant will be achieved to put forth potential isotope fractionation.

Acknowledgements

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V.3. Clinical study protocol

Étude pharmacocinétique du métabolisme de la nicotine après consommation de tabac fumé et non-fumé.

Laboratoire Suisse d'Analyse du Dopage (LAD)
Centre Universitaire Romand de Médecine Légale (CURML)
Polyclinique Médicale Universitaire (PMU)
Centre Hospitalier Universitaire Vaudois (CHUV)
Université de Lausanne
Directeur : Prof. Patrice Mangin

1. Titre de l'étude

Étude pharmacocinétique du métabolisme de la nicotine après utilisation de tabac fumé et non-fumé.

2. Date de l'envoi du protocole et de début de l'étude

Envoi du protocole : **janvier 2012**
Début de l'étude : **février 2012**
Fin prévue de l'étude : **mai 2012**

3. Cadre de l'étude

3.1 Investigateurs

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Investigateurs :

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- **François Marclay, MSc,** criminaliste, doctorant au LAD.
- **Carole Clair-Willi, MD,** médecin, IMEC, IUMSP, DUMSC.
- **Matthias Schubert, MD,** médecin-assistant, PMU.
- **Cong Khanh Huynh, Dr. ès Sc.**, ingénieur chimiste, IST, DUMSC.

3.2 Promoteur de l'étude

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3.3 Lieux de l'étude et responsables

Coordinateur principal : Martial Saugy, Dr. ès Sc., Directeur du LAD

Cette étude se déroulera sur deux sites :

- Laboratoire Suisse d'Analyse du Dopage (LAD)
  Centre Universitaire Romand de Médecine Légale (CURML)
  Département Universitaire de Médecine Sociale et Communautaire (DUMSC)
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- Policlinique Médicale Universitaire (PMU)
  CHUV
  Rue du Bugnon 44
  1011 Lausanne
  Responsable local : Jacques Cornuz, Prof., Directeur et Médecin-chef de la PMU

Une collaboration est également prévue dans le cadre de cette étude clinique avec l'institut suivant :

- Institut Universitaire Romand de Santé au Travail (IST)
  Département Universitaire de Médecine Sociale et Communautaire (DUMSC)
  CHUV
  Rue du Bugnon 19
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  Responsable local : Cong Khanh Huynh, Dr. ès Sc.

4. Perspectives de l'étude

4.1 Etat des connaissances

La consommation de tabac est un problème de santé publique mondial causant un vaste éventail de maladies dont les taux de mortalité sont parmi les plus élevés. Malgré tout, la popularité du tabac consommé sous une forme ou une autre demeure importante. Cela s'explique non seulement par des propriétés pharmacologiques de la nicotine propices à un usage récréatif, mais également par un pouvoir addictif très fortement prononcé.

Afin de limiter l'étendue de ce fléau, notamment les effets néfastes de la fumée passive, une politique de bannissement de la fumée dans les lieux publiques tend

doucement à s'imposer de part le monde. Dès lors, l'industrie du tabac promeut depuis quelques années la consommation de produits du tabac sans fumée (smokeless tobacco), en particulier le snus. En effet, ces poches à placer entre la lèvre et la gencive constitueraient selon eux une alternative au tabac fumé à la fois sûre pour la santé et consommable dans les lieux publics. Outre-Atlantique, la promotion de tels produits est souvent assurée par des sportifs professionnels. Cette propagande occulte les risques réels encourus par la consommation de snus dont la popularité semble grandissante. Le système respiratoire n'est certes pas endommagé, mais de part la quantité de composés cancérigènes qu'il contient, les cancers de la cavité buccale et de l'œsophage sont très significativement favorisés et celui du pancréas favorisé également. De même, des problèmes cardiaques et des lésions graves des tissus de la muqueuse buccale résultent de sa consommation. Enfin, le pouvoir addictif de ce type de produit égale celui du tabac fumé.

Sa consommation s'est naturellement propagée au sein de la communauté sportive pour plusieurs raisons. En effet, la nicotine du tabac non-fumé présente des propriétés stimulantes et relaxantes dont les effets sur la performance sont très intéressants. **Elle permet notamment d'inhiber la sensation de faim, de stimuler la sécrétion d'hormones anti-diurétiques ainsi que de faciliter la mise en action par un accroissement de la fréquence cardiaque et de la pression artérielle. La nicotine a également pour effet de diminuer l'anxiété, d'augmenter la concentration, d'accentuer la vigilance, d'intensifier la rapidité des réflexes et la vivacité, de stimuler et modifier les activités sensorielles et motrices, et d'optimiser la réponse au stress. Ces effets peuvent être bénéfiques à la pratique de sports de précision ou demandant une grande intensité.** De plus, le tabac non-fumé permet d'éviter une diminution de la capacité pulmonaire dont l'effet sur la performance serait négatif. La nicotine n'apparaissant ni dans la Liste des Substances Interdites 2011 (la Liste) de l'Agence Mondiale Antidopage (AMA), ni dans le Programme de Surveillance 2011, cette substance présente donc un intérêt particulièrement bénéfique à la pratique sportive. Jusque récemment, seules des informations informelles indiquaient la propagation de ce phénomène, initié par les sportifs Scandinaves et Nord-Américains, en particulier dans les sports d'hiver tels que le hockey sur glace ou le ski alpin. Afin de mesurer l'étendue du phénomène, le Laboratoire Suisse d'Analyse du Dopage (LAD) a réalisé deux études de prévalence successives, en 2009 et 2010. Lors des Championnats du Monde de Hockey sur Glace 2009 ayant pris place en Suisse, des traces de nicotines ou/et de ses métabolites furent décelées dans la totalité des échantillons urinaires. De même, une exposition dans les 3 jours avant la compétition par la consommation de tabac ou par la fumée passive fut relevée pour ~80% des athlètes. Enfin, la consommation active de tabac juste avant ou pendant un match fut mise en évidence pour ~50% des athlètes. Compte tenu des effets néfastes du tabac fumé sur l'organisme et les effets bénéfiques de la nicotine sur la performance, l'hypothèse

d'une large consommation de tabac non-fumé, en particulier de snus, apparut comme très fortement probable. La seconde étude s'intéressa donc à mesurer ce phénomène à large échelle, en impliquant l'ensemble des contrôles antidopage urinaires durant une année de 2010 à 2011 pour un total de 2'185 échantillons de 43 sports différents. En se focalisant sur le hockey sur glace, ski alpin, snowboard, biathlon, ski de fond, football, basketball, volleyball, rugby, football Américain, lutte et gymnastique, la consommation de tabac avant ou pendant la compétition fut à nouveau mise en évidence dans une proportion d'échantillons alarmante. En effet, une utilisation active fut déterminée pour environ 20% à 55% des athlètes de ces disciplines, alors que la prévalence de la fumée dans le monde atteint ~25%. Ces deux études constituent à ce jour les seules sources d'information scientifiques attestant de l'étendue actuelle de ce phénomène.

Toutefois, alors que la nicotine satisfait aux trois critères d'inclusion d'une substance à la Liste des Interdictions, à savoir améliorer la performance, présenter un risque pour la santé et véhiculer une image négative du sport, celle-ci n'y figure toujours pas. A l'heure actuelle, cette inclusion se heurte à un point déterminant, soit l'absence d'outils analytiques et chimiométriques permettant de distinguer la consommation récréative de tabac fumé de l'attitude dopante par la consommation de tabac non-fumé.

Néanmoins, suite aux travaux de recherche du LAD, la nicotine a été incluse au Programme de Surveillance 2012 au début Octobre, rendant cette étude clinique pertinente.

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4.3 But de l'étude

Selon le mode d'absorption de la nicotine (voies aériennes ou au travers de la muqueuse buccale), ses propriétés pharmacocinétiques pourraient présenter des variations significatives. En effet, de telles différences ont pu être observées lors de l'utilisation de patchs transdermiques, comparativement à la consommation de cigarette. Dès lors, cette étude pilote s'intéresse à la possibilité de distinguer de consommation du tabac fumé de celle du tabac non-fumé, en particulier celle du snus, par la mise en évidence de variations du profil métabolique de la nicotine et d'autres composés du tabac ou issus de la combustion de ce dernier.

4.4 Objectifs

Dans un premier temps, cette étude pilote devrait permettre d'établir le profil métabolique de la nicotine et de ses métabolites sous sa forme fumée ou non-fumée, mais également d'autre substances présentes dans le tabac, telles des alcaloïdes mineurs et des nitrosamines spécifiques ainsi que des substances issues de la combustion telles les hydrocarbones aromatiques polycycliques. Vraisemblablement, l'absorption de ces composés par les voies aériennes devrait présenter des variations significatives en termes de propriétés pharmacocinétiques comparativement à l'absorption au travers de la muqueuse buccale.

Dans un deuxième temps, ces profils métaboliques urinaires seront exploités afin de développer des outils d'analyse chimiométriques permettant de distinguer une forme ou l'autre de consommation. La mise en évidence de biomarqueurs, traduisant des variations de concentrations ou de rapports de concentrations, devrait permettre une détermination à l'aveugle du type de consommation d'un sujet.

Sachant les effets néfastes de la fumée sur la performance sportive, la possibilité de différencier la consommation de tabac fumé de celle de snus permettra de distinguer une consommation sociale d'une pratique potentiellement dopante. En s'appuyant sur

de telles bases scientifiques et analytiques, l'AMA pourra enfin considérer l'ajout de la nicotine à la Liste des Substances Interdites.

De plus, au-delà du caractère répressif d'une telle inclusion, celle-ci représenterait un outil de prévention de premier ordre afin d'empêcher l'initiation d'une addiction à la nicotine pendant la carrière sportive et son développement ultérieur, avec les risques sanitaires graves que cela implique.

4.5 Justification de l'étude

Malgré une toxicité et des propriétés cancérigènes connues de tous, la consommation mondiale de tabac est responsable de plus de 5 millions de décès chaque année, un chiffre qui devrait croître jusqu'à 8.3 millions en 2030. Le tabac contribue très fortement aux quatre principales causes de décès, notamment les maladies cérébrovasculaires et pulmonaires. En outre, le cancer de la trachée, des bronches et des poumons est un problème de santé majeur auquel la consommation de tabac est significativement corrélée.

Par conséquent, le traitement de la dépendance à la nicotine est une préoccupation d'ordre mondial qui peut être abordée selon différents angles. En effet, l'aide à l'abandon de la fumée par suivi médical ou pharmacothérapie, la prévention de l'initiation à la consommation, la réduction de l'exposition à la fumée passive dans les lieux publics et l'augmentation des taxes sont autant de mesures visant à la réduction des risques liés à la fumée. À travers le monde, l'interdiction de fumer dans les lieux publics gagne en popularité, avec pour conséquence une diminution significative des risques dus à l'exposition à la fumée active et passive, tout en favorisant la cessation de la consommation. En réaction à cet état de fait, l'industrie du tabac tend à diversifier sa gamme de produits afin de contourner une telle législation dont les préjudices économiques sont évidents. Dès lors, les campagnes publicitaires pour les produits nicotiniques sans fumée, en particulier pour le snus, se multiplient de par l'Europe et l'Amérique du Nord.

Cependant, des propriétés addictives similaires ont été observés en comparant la nicotine fumée et sans fumée, ce qui favorise sérieusement une initiation ultérieure au tabagisme. En outre, la consommation de tabac sans fumée à elle seule favorise sensiblement les risques de cancers de la cavité buccale, de l'œsophage et potentiellement du pancréas en raison de la présence de plus de 28 substances cancérigènes, mais aussi de maladies cardiaques et de lésions des tissus buccaux.

Le monde sportif est lui aussi touché de plein fouet par la popularité grandissante de ce genre de produits, que son usage soit social ou à caractère dopant. A ce titre, la nicotine présente des effets stimulants et relaxants conjoints, pouvant potentiellement améliorer la performance, particulièrement sous la forme de snus.

Toutefois, en dépit de satisfaire les trois critères d'inclusion édicté par l'AMA, la nicotine ne figure toujours pas dans la Liste des Interdictions et vient tout juste d'être incluse au futur Programme de Surveillance 2012. Cette substance peut non seulement améliorer la performance sportive, mais représente également un risque majeur pour la santé et ternit l'esprit du sport en raison de l'image négative véhiculée par la consommation de tabac. Dès lors, l'obstacle crucial à l'inclusion de cette substance dans la Liste est l'absence d'études pharmacocinétiques du métabolisme de la nicotine selon une forme ou l'autre de consommation et de méthodes analytiques et chimiométriques permettant leur distinction. En effet, à l'heure actuelle il n'existe aucune étude comparative du métabolisme de la nicotine selon ces deux modes de consommation. Ce domaine demande à être exploré et fournirait non seulement des informations de premier ordre pour la lutte contre le dopage, mais également des données précieuses pour la recherche en tabacologie afin de mieux comprendre et traiter les problèmes liés à la consommation de produits nicotiniques sans fumée.

De plus, cette inclusion présenterait un aspect préventif dont l'importance dépasse le simple caractère répressif. En effet, les risques pour la santé suite à la consommation de tabac sous n'importe quelle forme sont tels que cette démarche permettrait d'éviter l'initiation à cette pratique durant la pratique sportive. Sachant que la consommation de tabac non-fumé favorise très significativement le passage à la cigarette, cette inclusion aiderait à la résolution de ce problème de santé publique au sein de la population sportive.

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5. *Plan général*

Cette étude pilote sur la prise de nicotine va être menée sur trois sites. La séance d'information préliminaire avec les sujets aura lieu au LAD, la visite d'inclusion à la PMU, l'administration de nicotine ainsi que la collecte d'échantillons biologiques (sang et urine) sera effectuée à l'IST sous la direction du Prof. Jacques Cornuz et l'analyse des échantillons se fera au LAD sous la direction du Dr. Martial Saugy.
La nicotine du tabac fumé et non-fumé sera administrée à des volontaires masculins sains (n = 20) recrutés par voie d'annonce (Annexe 1). Un formulaire de recrutement (Annexe 2) sera envoyé par e-mail par François Marclay aux volontaires ayant signifié leur volonté de participer à cette étude clinique. Ces derniers nous retourneront le formulaire rempli pour être contacté à la séance d'information. Ceci permettra de contacter uniquement les volontaires répondant aux critères de l'annonce de recrutement. Les volontaires seront convoqués à une séance d'information qui sera donnée par François Marclay et qui se déroulera au LAD. Cette séance a pour but de donner les indications précises quand au déroulement de l'étude. La feuille d'information aux volontaires (Annexe 3) leur sera donnée, ainsi que le formulaire de consentement (Annexe 4). Les volontaires transmettront à François Marclay le formulaire de consentement dans un délai de 3 jours suivant la séance d'information, afin qu'ils bénéficient d'un temps nécessaire de réflexion.

Dès que les feuilles de consentement seront retournées à François Marclay dûment signées, les volontaires seront convoqués pour la visite d'inclusion qui sera effectuée

par un docteur de la PMU (Dr. Carole Clair-Willi). Lors de cette visite, les paramètres d'inclusion et d'exclusion seront vérifiés à l'aide d'un questionnaire (Annexe 5) et un numéro de sujet sera attribué de manière aléatoire à chaque volontaire. Ce numéro sera reporté dans le cahier d'observation (CRF, annexe 6) et seul l'investigateur principal et son personnel, tous soumis au secret professionnel auront accès aux données personnelles des sujets.

Le protocole permettant de suivre le métabolisme de la nicotine, de ses métabolites, des alcaloïdes mineurs et nitrosamines spécifiques du tabac, et des hydrocarbones aromatiques polycycliques est résumé dans le tableau 1.

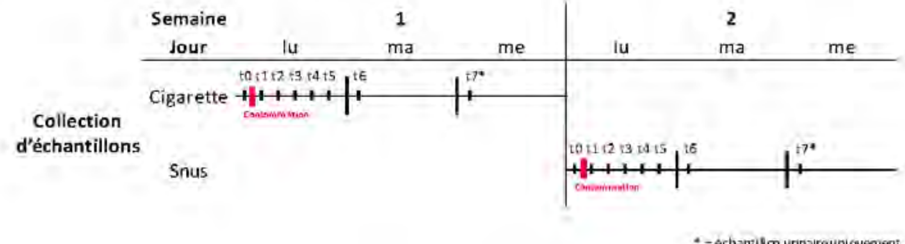
Afin de pouvoir déterminer précisément le métabolisme de ces différentes substances, il sera demandé aux volontaires de ne pas consommer de nicotine durant les 3 jours précédents le début de chaque phase et durant les 3 jours suivant l'administration de nicotine.

Lors du jour 1 de l'étude (lundi), une cigarette standard (Parisienne Jaune) de 0.8 g de tabac contenant 0.7 mg de nicotine sera fumée par les volontaires dans un environnement contrôlé à l'IST. La récolte d'échantillons sanguins sera effectuée aux temps t0 = 0h (avant la prise), t1 = 2h, t2 =4h, t3 = 6h, t4 = 8h, t5 = 10h et t6 = 24h après la prise. De même, la récolte d'échantillons urinaires sera effectuée aux temps t0 = 0h (avant la prise), t1 = 2h, t2 =4h, t3 = 6h, t4 = 8h, t5 = 10h, t6 = 24h et t7 = 48h après la prise.

Puis, une semaine de pause sera observée afin de garantir une complète élimination des substances d'intérêts.

Lors du jour 8 de l'étude, une poche de snus de 24 g de tabac contenant 8 mg de nicotine sera administrée aux volontaires pendant une durée de 30min à l'IST. La récolte d'échantillons sanguins sera effectuée aux temps t0 = 0h (avant la prise), t1 = 2h, t2 =4h, t3 = 6h, t4 = 8h, t5 = 10h et t6 = 24h après la prise. De même, la récolte d'échantillons urinaires sera effectuée aux temps t0 = 0h (avant la prise), t1 = 2h, t2 =4h, t3 = 6h, t4 = 8h, t5 = 10h, t6 = 24h et t7 = 48h après la prise.

Tableau 1 : Protocole de l'étude clinique



Désagréments pour les volontaires

Le protocole va se dérouler sur une période de 2 semaines. L'administration de nicotine sera effectuée 2 lundi matins. Pour ces deux journées, la récolte de sang et d'urine sera effectuée au centre d'étude après 0, 2, 4, 6 8 et 10h. Les volontaires passeront donc ces deux journée dans un environnement contrôlé. De même, l'échantillon sanguin à 24h sera récolté le lendemain matin au centre d'étude. Par la suite, les échantillons urinaires à 24h et 48h seront récoltés directement par les sujets à l'aide de matériel mis à disposition et ne devrait pas nuire de façon significative à une organisation normale.

Les volontaires seront dédommagés à hauteur de Frs. 500.- qu'ils recevront lors de l'examen médical de sortie. Tout sujet est averti de la possibilité de retirer son consentement à tout moment quelle qu'en soit la raison et sans avoir à la justifier.

Si un volontaire, pour une raison quelconque, doit mettre un terme à sa participation au protocole, il devra obligatoirement subir l'examen médical de sortie et sera dédommagé au prorata de son temps de participation.

L'identité des sujets ne sera connue que par les responsables de l'étude au sein de chaque unité. L'investigateur responsable garanti la confidentialité des données obtenues lors de l'examen préliminaire et des analyses propres à l'étude.

Le protocole est couvert par l'autoassurance en responsabilité civile de l'Etat de Vaud (Annexe 4).

Devenir des échantillons

Toutes les données seront reportées dans le cahier d'observation (CRF, annexe 7) et archivées au LAD. Les échantillons seront anonymisés et conservés à -20°C au LAD. A la fin de l'étude, la clé d'anonymisation sera détruite. Une fois que les analyses pour les besoins de cette étude auront été réalisées, ces échantillons pourront servir de contrôles positifs pour des analyses de contre-expertise pour mettre en évidence et confirmer un potentiel dopage à la nicotine. Après 10 ans, les échantillons seront détruits.

6. Sélection des sujets

Une visite médicale préalable sera effectuée par un médecin de la PMU (Dr. Carole Clair-Willi) lors de la sélection des sujets. Les informations relatives à l'étude ainsi que le formulaire de consentement leur seront donnés lors de ce même entretien.

Les volontaires seront recrutés par voie d'annonces à la Faculté de Biologie et Médecine de Lausanne ainsi qu'à l'Institut des sciences du sport de l'Université de Lausanne (Annexe 1) et seront sélectionnés selon les critères suivant :

Critères d'inclusion :
20 sujets mâles d'origine caucasienne âgés de 20 à 30 ans, fumeurs occasionnels **(1 à 3 fois par semaine)** en bonne santé, ayant une activité physique régulière **(pratique sportive 1 fois au moins par semaine)** et possédant un index de masse corporel (IMC) compris entre 18 et 30 (IMC = poids / taille^2).

Critères d'exclusion :
Sont exclus de l'étude les sujets présentant une ou plusieurs des caractéristiques suivantes :

- Prise régulière de tabac (4x ou plus par semaine)
- Prise régulière de drogues ou d'alcool (2x ou plus par semaine)
- Prise de médicaments
- Prise de substituts nicotiniques (patch, gomme, pastille, inhaleur,...)
- Ulcères gastroduodénal, colites ulcéreuses, maladie de Crohn
- Maladies cardiovasculaires (arythmie, insuffisance cardiaque manifeste ou latente, hypertension artérielle)
- Maladies pulmonaires (broncho-pneumopathie chronique obstructive, asthme)
- Maladies néoplasiques (cancer)
- Maladies infectieuses
- Insuffisance rénale
- Troubles hépatiques
- Allergies
- Migraine
- Epilepsie

De plus, les personnes affiliées à l'un des centres impliqués dans cette étude ne pourront pas participer en tant que sujet.

7. Méthodes d'investigations

Les administrations de nicotine sous forme de cigarette ou de snus seront supervisées au sein de l'IST du CHUV.

Les échantillons sanguins (minimum 5 mL de sérum) ainsi que les échantillons d'urine (minimum 50 mL) seront collectés directement par le personnel de ce centre dans des récipients prévus à cet effet lors des deux journées d'administration ainsi qu'après 24h. Les échantillons urinaires suivants seront récoltés directement par les volontaires à l'aide de matériel mis à disposition. Les urines seront rapportées au LAD le plus rapidement possible afin d'être aliquotées puis congelées à –20°C en attendant d'être analysées. La clef d'anonymisation sera détruite à la fin de l'étude.

Le profil métabolique de la nicotine, de ses métabolites, des alcaloïdes mineurs et nitrosamines spécifiques du tabac, et des hydrocarbones aromatiques polycycliques sera mesuré dans le sang et l'urine à l'aide de méthodes de chromatographie liquide couplée à la spectrométrie de masse préalablement validées au LAD. De même, la créatinine sera mesurée dans l'urine par chimie clinique et la gravité spécifique à l'aide d'un réfractomètre afin de normaliser les concentrations des composés d'intérêt. Enfin, le pH sera estimé avec un papier pH et le volume de chaque échantillon urinaire sera relevé.

8. Surveillance médicale

Ce projet de recherche concerne un groupe de sujets sains, volontaires, ayant une activité physique régulière mais ne pratiquant pas une activité sportive de haut niveau. Les sujets d'étude seront tous des fumeurs occasionnels.

Les risques éventuels liés à une administration de cigarette ou de snus en monodose, correspondant à respectivement 0.7 mg et 8 mg de nicotine, sont extrêmement faibles si les critères d'exclusion ont bien été suivis lors de l'entretien des sujets. Les volontaires bénéficieront d'un suivi durant l'étude et d'un encadrement médical en cas d'apparition d'effets secondaires. Si un sujet présente des effets indésirables suite à l'administration de nicotine par l'une des voies décrites, il sera immédiatement écarté de l'étude et sera alors suivi jusqu'à une stabilisation de son état et un retour à la normale.

Les volontaires pourront contacter à toute heure le médecin responsable pendant la période de l'étude. Celui-ci sera prêt à répondre à leurs questions et à les recevoir en cas urgence.

9. Rôle du personnel soignant

Le personnel de l'IST sera impliqué dans l'administration de cigarette et de snus. La supervision de la collecte des échantillons sanguins et urinaires sera assurée par l'un des investigateurs de l'étude.

10. Produits

Cigarette

Pour cette étude, des cigarettes de la marque Parisienne Jaune ont été sélectionnées en raison de leur très grande popularité chez les fumeurs en Suisse et leur contenu moyen en nicotine. Ces cigarettes contiennent 0.7 mg de nicotine pour 0.8 g de tabac. La teneur en goudron annoncée est de 8 mg.

Lors de l'inhalation de fumée, la nicotine est rapidement absorbée par l'épithélium alvéolaire. De part le flux sanguin élevé des capillaires pulmonaires, l'absorption par la circulation systémique est facilitée, distribuant la nicotine au travers des organes très rapidement. Dès lors, la nicotine traverse la membrane hémato-encéphalique afin d'attendre le cerveau en moins de 20 s. et la concentration plasmatique atteint un pic après ~5 min. Après cessation de la fumée, la nicotine plasmatique et urinaire est éliminée dans les 24 h du fait de sa demi-vie correspondant à $t_{1/2} \approx$ 120 minutes.

Snus
Des poches de snus General Portion de la marque Swedish Match ont été sélectionnées de part leur très grande popularité chez les consommateurs Européens et leur contenu moyen en nicotine. Ces poches contiennent 8 mg de nicotine pour 24 g de tabac.
En raison du pH alcalin auquel est conditionné le tabac contenu dans les poches, la nicotine est rapidement absorbée au travers de la muqueuse buccale. De plus, la celle déglutie est absorbée au niveau de l'intestin grêle. Au contraire de la nicotine inhalée, l'absorption sous forme de snus présente un délai et atteint un pic plasmatique après ~30min.

11. Evaluation des risques et enjeux éthiques

11.1 Toxicité des substances

Cigarette
De très nombreuses études cliniques ont été réalisées sur la toxicité de la cigarette. Les effets indésirables discutés ci-dessous ont surtout été observés sur des expositions de longue durée (plusieurs mois).
Du à la pyrolyse du tabac, la fumée de la cigarette contient plus de 4'000 composés, dont au moins 60 ont été identifiés comme cancérigènes. L'exposition répétée à ces substances, notamment par le très haut pouvoir addictif de la nicotine, est significativement corrélée au développement de nombreuses maladies et cancers. En effet, la fumée est la contribution la plus majeure aux quatre causes de mortalités non-accidentelle les plus répandues à travers le monde, à savoir les cardiopathies ischémiques, les maladies cérébrovasculaires, les infections des voies respiratoires inférieures et les maladies pulmonaires obstructives chroniques. De plus, les cancers de la trachée, des bronches et des poumons sont fortement favorisés.

Snus

De même, de nombreuses études cliniques ont été réalisées sur la toxicité du snus et du tabac non-fumé en général. A nouveau, les effets indésirables discutés ci-dessous ont surtout été observés sur des expositions de longue durée (plusieurs mois).

Parmi les nombreux constituants du tabac sous forme de snus, plus de 28 composés cancérigènes ont peut être identifiés. L'exposition répétée à ces substances, notamment due au très haut pouvoir addictif de la nicotine, est corrélée aux cancers de la cavité buccale, de l'œsophage et du pancréas. De même, des problèmes cardiaques et des lésions graves des tissus de la muqueuse buccale résultent de sa consommation.

11.2 Risque liés à la consommation durant l'étude

Dans le cadre de cette étude, la consommation cigarette et de snus se limite à une seule unité. L'exposition aux différents composés carcinogènes, et toxiques d'une manière générale, est donc négligeable. Ceci est particulièrement vrai sachant que les volontaires sont tous des fumeurs occasionnels s'exposant déjà de manière répétée à ces substances. De même, le risque d'addiction à la nicotine et l'augmentation de la consommation de cigarettes ou snus suite à cette étude est négligeable pour des raisons similaires. Le facteur risque est extrêmement faible.

12. Considérations financières

Le financement nécessaire à la réalisation de cette étude provient des fonds propres du LAD et de la PMU ainsi que de fonds de recherche réguliers fournis par Antidoping Switzerland (ADS) pour la recherche et le développement.

- Rétribution des volontaires		Frs.	10'000.-
- Charges salariales		Frs.	20'000.-
- Frais administratifs et logistiques		Frs.	7'000.-
- Supervision médicale		Frs.	3'000.-
- Produits :	Cigarette (2 paquets)	Frs.	15.-
	Snus (2 boîtes)	Frs.	15.-
- Analyses		Frs.	50'000.-
	Total	***Frs.***	***90'030.-***

Les Frs. 50'000.- mentionnés sous la rubrique « analyses » couvrent les frais d'analyses et de fonctionnement impliquant le personnel de l'institut.

13. Gestion des données

Pour chaque participant à l'étude, un dossier médical regroupant les données administratives et médicales sera constitué. Ce dossier sera gardé sous clé. Chaque participant sera identifié par un numéro d'inclusion aléatoire déterminé par un médecin de la PMU (Dr. Carole Clair-Willi), permettant de lier les différentes données récoltées entre elles. Les données non informatisées récoltées pendant l'étude seront relevées par l'investigateur principal et son personnel, tous soumis au secret professionnel, dans un cahier d'observation qui sera conservé indépendamment du dossier médical constitué. A la fin de l'étude, les dossiers seront archivés dans une armoire sous clé pendant 10 ans. L'accès aux données sera limité au personnel soignant impliqué dans l'étude. Enfin, la clé d'anonymisation sera détruite afin que l'anonymat soit conservé. Les échantillons seront aliquotés et conservés pendant 10 ans avant destruction. Ces échantillons pourront servir de contrôle positif en cas d'analyse de contre-expertise pour confirmer un dopage potentiel à la nicotine.

14. Signataires

	Signature :	Date :
Dr. Martial Saugy, Directeur scientifique Laboratoire Suisse d'Analyse du Dopage	…………………………………………………	
Prof. Jacques Cornuz, Médecin-chef Directeur, PMU	…………………………………………………	
Prof. Patrice Mangin, Médecin Directeur, DUMSC	…………………………………………………	

15. Annexes

Annexe 1. Annonce de recrutement des sujets

Le Laboratoire Suisse d'Analyse du Dopage

recherche

20 volontaires masculins de 20 à 30 ans
en bonne santé et fumeurs occasionnels
en vue de participer à une étude visant à étudier le métabolisme de la nicotine après consommation de tabac fumé et non-fumé.

Un défraiement de 500.- sera octroyé par participant.

Pour information, merci de contacter:

François Marclay
Laboratoire Suisse d'Analyse du Dopage (LAD)
Centre Universitaire Romand de Médecine Légale (CURML)
Centre Hospitalier Universitaire Vaudois (CHUV)

Tél: +41 (0)21 314 73 30
E-mail: Francois.Marclay@chuv.ch

Annexe 2. Formulaire de recrutement

Laboratoire Suisse d'Analyse du Dopage
Institut Universitaire de Médecine Légale
Chemin des Croisettes 22
1066 Epalinges

Lausanne, le 23 août 2013

Étude pharmacocinétique du métabolisme de la nicotine
après consommation de tabac fumé et non-fumé.

Formulaire de recrutement

Sujet n° ___ Date: ___/___/___

Critères d'inclusion:

		Conforme	Non-conforme
Age:______(20 à 30)		□	□
Poids:______			
Taille:______			
IMC: poids/taille² =______	(18 à 30)	□	□
Fumeur occasionnel **(1 à 3 fois par semaine)**		□	□
Activité physique régulière (pratique sportive 1 fois au moins par semaine)		□	□

Données personnelles:
Nom:
Prénom:
Adresse:
Tél. :
E-mail:

Annexe 3. Formulaire d'information

Laboratoire Suisse d'Analyse du Dopage
Institut Universitaire de Médecine Légale
Chemin des Croisettes 22
1066 Epalinges

Lausanne, le 23 août 2013

Étude pharmacocinétique du métabolisme de la nicotine après consommation de tabac fumé et non-fumé.

Feuille d'information

Monsieur,

1. Sélection des participants à l'essai clinique

Nous vous proposons de participer à une étude clinique dans le domaine de la lutte anti-dopage dont le but est d'obtenir une information plus large sur le métabolisme de la nicotine et des constituants du tabac après exposition sous formes inhalée (cigarette) et orale (snus). Cette étude se déroulera à la Polyclinique Médicale Universitaire (PMU) du CHUV pour la partie médicale, à l'Institut Romand de Santé au Travail (IST) pour l'administration de nicotine ainsi que la collecte d'échantillons biologiques (sang et urine) et au Laboratoire Suisse d'Analyse du Dopage (LAD) pour le traitement des échantillons.

2. Objet de l'étude

Afin de contrer le bannissement de la fumée dans les lieux publics, l'industrie du tabac promeut la consommation de produits du tabac sans fumée (smokeless tobacco), en particulier le snus (poche de tabac à placer entre la lèvre et la gencive).

Bien que les propriétés relaxantes et stimulantes de la nicotine puissent améliorer la performance, elle n'apparait pas dans la Liste des Substances Interdites 2012 (la Liste) de l'Agence Mondiale Antidopage (AMA). Cet état de fait rend particulièrement intéressant la consommation de tabac non-fumé à but de dopage, de par ces propriétés et l'absence d'effets indésirables sur la capacité pulmonaire.

Cet essai clinique propose d'étudier le métabolisme de la nicotine et autres composés du tabac absorbés par consommation de tabac fumé (cigarette) et non-fumé (snus). Ces analyses permettront de développer des outils statistiques pour interpréter les résultats de contrôles antidopage, afin de distinguer consommateurs de cigarettes et de snus, dans le but d'informer sur une potentielle pratique dopante.

3. Informations générales sur l'étude

Cet essai clinique, incluant 20 volontaires masculins fumant de manière occasionnelle (1 à 3 fois par semaine), sera effectué en vous administrant de la nicotine sous deux formes. Tout d'abord, il vous sera demandé de fumer une cigarette standard de 0.8 g de tabac contenant 0.7 mg de nicotine. La semaine suivante, une poche de snus de 24 g de tabac contenant 8 mg de nicotine vous sera administrée pendant une durée de 30min.

Des analyses seront effectuées sur le sang et l'urine pour évaluer précisément le métabolisme de la nicotine et des composés du tabac après chaque administration.

Cette étude est réalisée conformément aux lois suisses en vigueur et dans le respect de principes reconnus au plan international. Le protocole de cette étude a reçu l'avis positif de la Commission d'éthique de la recherche sur l'être humain du Canton de Vaud, en date du XX/XX/2012.

4. Caractère volontaire de la participation

Votre participation à cet essai est volontaire. Vous pouvez à tout moment revenir sur votre consentement à prendre part à cette étude, sans être tenu de justifier votre décision. En cas de révocation de votre consentement, les données recueillies jusqu'alors continueront à être utilisées. Le cas échéant, les échantillons prélevés dans le cadre de l'essai (sang et urine) seront détruits.

Si vous révoquez votre consentement, vous serez soumis à un examen médical final pour votre propre sécurité.

5. Déroulement de l'étude

Cette étude est une étude dite pilote, qui vise à mettre en évidence une prise de nicotine par la consommation de snus chez l'homme sain, dans le but de la lutte contre le dopage.

Cette étude clinique préliminaire sur la prise de nicotine va être menée sur trois sites. L'entretien préliminaire sera réalisé à la Polyclinique Médicale Universitaire (PMU) du CHUV sous la direction du Prof. Jacques Cornuz, l'administration de nicotine ainsi que la collecte d'échantillons biologiques (sang et urine) à l'Institut Romand de Santé au Travail (IST) sous la direction du Prof. Jacques Cornuz également et le traitement des échantillons au Laboratoire Suisse d'Analyse du Dopage (LAD) sous la direction du Dr. Martial Saugy.

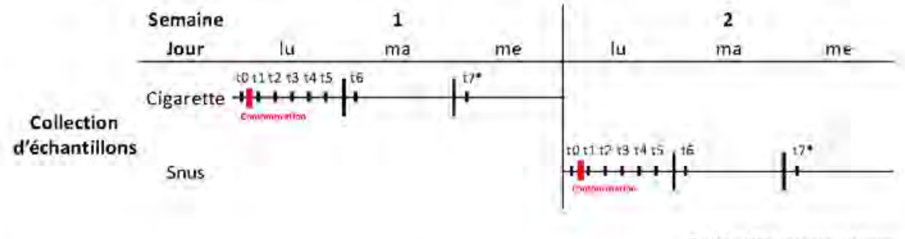
Dès que les feuilles de consentement que vous avez obtenues lors de la séance d'information au LAD seront retournées à François Marclay dûment signées, vous

serez convoqués par e-mail ou par téléphone pour la visite d'inclusion qui sera effectuée par le Dr. Carole Clair-Willi à la PMU du CHUV.

Inclusion : Une visite médicale préalable sera effectuée en début d'étude avant inclusion et les résultats vous seront transmis.

Période de traitement : L'étude se déroulera sur une période de 2 semaines comprenant 2 phases principales, représentées dans le tableau ci-dessous.

Tableau 1 : Protocole de l'étude clinique



I. Lors du jour 1 de l'étude (lundi), une cigarette standard (Parisienne Jaune) de 0.8 g de tabac contenant 0.7 mg de nicotine sera fumée dans un environnement contrôlé. La récolte d'échantillons sanguins (minimum 5 mL de sérum) sera effectuée aux temps t0 = 0h (avant la prise), t1 = 2h, t2 =4h, t3 = 6h, t4 = 8h, t5 = 10h et t6 = 24h après la prise. De même, la récolte d'échantillons urinaires (minimum 50 mL) sera effectuée aux temps t0 = 0h (avant la prise), t1 = 2h, t2 =4h, t3 = 6h, t4 = 8h, t5 = 10h, t6 = 24h et t7 = 48h après la prise.

   Les récoltes du jour 1 seront effectuées directement au centre d'investigation et celle sanguine à t6 = 24h également lors de la visite le matin suivant (jour 2). Les échantillons urinaires à t6 = 24h et t7 = 48h seront récoltés par le volontaire chez lui à l'aide de matériel mis à disposition et déposés au centre d'investigation.

Puis, une semaine de pause sera observée afin de garantir une complète élimination des substances d'intérêts.

II. Lors du jour 8 de l'étude (lundi), une poche de snus de 24 g de tabac contenant 8 mg de nicotine sera administrée pendant une durée de 30min. La récolte d'échantillons sanguins et urinaires se déroulera exactement comme pour la phase I.

Logistique : L'administration de nicotine, de même que les prises de sang et récoltes d'urine seront effectuées le matin à l'IST. Vous serez pris en charge par les infirmières de la PMU du CHUV et François Marclay sera présent pour assurer le bon déroulement de l'étude clinique selon le protocole.

Le lundi de la première semaine, les échantillons de sang et d'urine seront collectés à 7h00 du matin et vous resterez à l'IST pour les collectes d'échantillons aux temps 2 et 4h après la prise (9h00 et 11h00, respectivement). Vous reviendrez pour 13h00 à l'IST pour la collecte des échantillons au point 6h et resterez sur place pour les points à 8 et 10h (15h00 et 17h00). Vous serez attendu le mardi matin à 7h00 pour collecter l'échantillon sanguin et rapporter l'échantillon urinaire correspondants au temps 24h, ainsi que le mercredi matin à 7h00 pour rapporter le dernier échantillon urinaire au temps 48h.

La deuxième phase débutera le lundi suivant et se déroulera de la même manière.

Il est à noter que vous récolterez vous-mêmes les urines produites à 24 et 48h après administration. Pour ce faire, vous recevrez un nombre suffisant de flacon de 100 mL que vous pourrez rapporter à l'IST le mardi et le mercredi.

Lors de cette étude, le nombre total de prises de sang veineux (1 tube de sérum 5 mL) effectuées par le personnel médical de la PMU sera de 24. Afin de faciliter les prises de sang, une veineflon vous sera posé avant la première collecte, réduisant le nombre total de piqures à 2. Les échantillons seront directement traités par un investigateur du LAD. La quantité totale de sang veineux prélevée sera de 120 mL pour une période de 2 semaines.

Examen de sortie : A la fin de l'étude, une visite médicale systématique sera effectuée. Les résultats vous seront transmis si vous le souhaitez. Si nécessaire, des examens de laboratoire complémentaires pourront être réalisés en cas d'apparition d'effets secondaires liés aux traitements.

6. Obligations incombant au participant

En tant que participant à l'essai clinique, vous êtes tenu :

- de suivre les instructions de votre investigateur et de vous conformer au plan de l'étude;
- d'informer précisément votre investigateur des effets indésirables constatés;
- d'informer votre investigateur d'un traitement auprès d'un médecin et de la prise de médicaments; font également partie des médicaments toutes les préparations que vous avez achetées vous-même, disponibles sans ordonnance et/ou rattachées à une médecine alternative (herbes, plantes, essences homéopathiques et

spagyriques, produits thérapeutiques asiatiques, denrées alimentaires spéciales et vitamines).

- afin de pouvoir mesurer précisément la nicotine absorbée par l'organisme durant cette étude, il est impératif de ne pas avoir consommé de nicotine durant les 3 jours précédents le début de chaque phase et durant les 3 jours suivant l'administration de nicotine. Tout manquement à cette règle conduira à l'exclusion du volontaire.

7. Avantages pour les participants

Participer à cette étude ne vous procurera aucun avantage, mais votre participiation pourra être une aide pour la lutte contre le dopage. En effet, la prise de nicotine pour la pratique sportive de haut niveau, mais aussi au niveau amateur, semble être un phénomène sérieux et grandissant. Cette problématique suscite une attention particulière au sein de l'AMA qui l'a ajoutée à son Programme de Surveillance 2012. Grace à votre participation, il sera peut-être possible de distinguer la prise de nicotine sous forme de tabac fumé de celle sous forme de tabac non-fumé.

8. Risques et désagréments

Les effets secondaires liés à la consommation chronique de tabac sont à exclure dans le cadre de cette étude car la consommation cigarette et de snus se limite à une seule unité. L'exposition aux différents composés carcinogènes ou/et toxiques est donc négligeable. De même, le risque d'addiction à la nicotine et l'augmentation de la consommation de cigarettes ou snus suite à cette étude est négligeable du fait de la faible dose de nicotine administrée de façon ponctuelle. Le facteur risque est donc extrêmement faible.

Toutefois, tout effet secondaire devra être signalé dès son apparition au Dr. Carole Clair-Willi (+41 (0)21 314 61 01) et l'administration sera provisoirement suspendue, si nécessaire.

L'étude se déroulera sous surveillance médicale stricte et en cas de problèmes médicaux engendrés par l'étude, la responsabilité civile de l'Etat de Vaud est engagée.

Si vous accepter de participer à cette étude, vous aurez la possibilité, à tout moment, d'interrompre volontairement l'étude sans que vous n'ayez à vous justifier.

9. Découvertes pertinentes

Le cas échéant, l'investigateur vous informera de toute découverte pouvant nuire à l'avantage ou à la sécurité de l'étude et donc influer sur votre consentement à participer à l'essai clinique.

Ces informations vous seront communiquées par écrit.

10. Confidentialité des données

Des données personnelles vous concernant sont recueillies pendant l'essai. Elles sont toutefois rendues anonymes et ne sont accessibles qu'à des spécialistes à des fins d'analyse scientifique. Toutes les données de l'étude seront anonymisées par système de code par le Dr. Carole Clair-Willi de la PMU et conservées de façon strictement confidentielle pendant 10 ans.

Les échantillons seront conservés au Laboratoire Suisse d'Analyse du Dopage. Une fois que les analyses pour les besoins de cette étude auront été réalisées, ces échantillons pourront servir de contrôles positifs pour des analyses de contre-expertise pour mettre en évidence et confirmer un dopage à la nicotine. Les échantillons seront détruits après 10 ans de conservation. Toutes les données personnelles seront reportées dans le cahier d'observation auquel seul l'investigateur principal et son personnel, tous tenus au secret professionnel, auront accès. Les données seront archivées à la fin de l'étude et la clé d'anonymisation sera détruite. Si l'étude devait être arrêtée prématurément, les données déjà recueillies pourront être utilisées et seront ensuite archivées.

Les spécialistes compétents du promoteur peuvent, dans le cadre de ce que l'on appelle un monitoring ou un audit, contrôler la procédure de réalisation de l'essai clinique. A ce titre, ils peuvent être amenés, tout comme les membres des autorités compétentes procédant à des inspections, à consulter les données brutes vous concernant. Reste que leur confidentialité est strictement garantie pendant toute la durée de l'essai clinique et lors des contrôles précités. Votre nom ne pourra donc en aucun cas être publié dans des rapports ou des publications qui découleraient de cet essai.

11. Frais

Les examens et les produits étudiés mentionnés dans cette information aux sujets de recherche sont gratuits. Ni vous, ni votre caisse-maladie n'avez à supporter quelque frais supplémentaire que ce soit qui découlerait de votre participation.

12. Rétribution des volontaires

Vous recevrez, pour votre participation à cet essai clinique un défraiement de 500.-. En cas de retrait avant la fin de l'étude, vous serez dédommagé au prorata du temps de participation à cette dernière.

13. Interruption involontaire de l'étude

Dans le cas où l'étude devait être interrompue par le médecin-investigateur, le promoteur de l'étude ou les co-investigateurs, un examen final devra être réalisé pour votre sécurité.

14. Répartition des dommages subis

Le CHUV s'engage, en tant que promoteur, à réparer tout dommage éventuel que pourriez subir dans le cadre de cet essai clinique.

Le protocole est couvert par l'autoassurance en responsabilité civile de l'Etat de Vaud.

Si, pendant ou à l'issue de l'essai clinique, vous devriez souffrir de problèmes de santé ou constater des dommages d'une autre nature, veuillez vous adresser au Prof. Jacques Cornuz, qui engagera pour vous la procédure requise.

15. Interlocuteurs

En cas d'urgence, d'incertitude ou d'événement inattendu ou indésirable survenant pendant ou après l'essai clinique, vous pouvez vous adresser à tout moment à la personne suivante :

Médecin-Investigateur : Prof. Jacques Cornuz, Directeur et Médecin-chef, PMU (+41 21 314 05 06, ou au piquet de tabacologie au +41 21 314 61 01)

Les investigateurs se tiennent à disposition des volontaires pour toutes informations complémentaires sur l'étude. Le protocole original est à leur disposition. Une copie des examens d'inclusion leur sera transmise sur demande auprès du Prof. Jacques Cornuz. L'investigateur principal se réserve le droit de contacter au besoin le médecin-traitant des volontaires uniquement sur leur accord préalable.
Par ailleurs, tous les investigateurs garantissent aux volontaires la confidentialité de tout ce qu'ils auront pu apprendre à leur sujet dans le cadre de l'étude.

Nous vous remercions par avance de votre intérêt pour cette étude et de votre investissement personnel pour la mener à bien.

Lausanne, le 23 août 2013

Dr. Martial Saugy Responsable scientifique et Directeur du LAD Tél: +41 (0)21 314 73 32	Prof. Jacques Cornuz Médecin-chef et Directeur de la PMU Tél: +41 (0)21 314 05 06

Annexe 4. Formulaire de consentement

Laboratoire Suisse d'Analyse du Dopage
Institut Universitaire de Médecine Légale
Chemin des Croisettes 22
1066 Epalinges

Lausanne, le 23 août 2013

Étude pharmacocinétique du métabolisme de la nicotine
après consommation de tabac fumé et non-fumé.

Formulaire de consentement

J'accepte volontairement et sans contrainte de prendre part à l'étude mentionnée ci-dessus et de collaborer pleinement à sa réalisation.

J'ai lu et compris la feuille d'information dans sa forme soumise à la commission d'éthique, et j'ai reçu du Prof. Jacques Cornuz des explications claires sur son but, son déroulement, ainsi que les risques possibles pour mon bien-être et pour ma santé.

Je suis actuellement en bonne santé et ne reçois aucun traitement médicamenteux. Je m'engage à répondre avec véracité aux questions de l'investigateur, en particulier quant à mon passé médical et mes habitudes (tabac, alcool, médicaments, drogues,...).
J'accepte qu'un examen clinique soit pratiqué afin de déterminer mon inclusion dans l'étude. J'autorise un éventuel contact entre les investigateurs et mon médecin traitant habituel :

☐ Oui ☐ Non

Je prends note que la confidentialité m'est garantie par les investigateurs, mais j'accepte que les informations récoltées à mon sujet dans le cadre de l'étude soient transmises, **sous forme anonymisée**, aux personnes impliquées dans l'étude (également liées pas le secret professionnel).

Je n'ai pas donné mon sang ni participé à une étude médicale durant les 2 derniers mois, et n'ai pris aucun médicament ou compléments alimentaires durant ces 2 derniers mois que je n'ai mentionné aux investigateurs.

Je prends note que les investigateurs assument les responsabilités de l'étude, mais m'engage de mon côté à suivre scrupuleusement leurs prescriptions.

Je demeure libre de me retirer de l'étude à tout moment et pour n'importe quelle raison. Si l'étude se termine prématurément, suite à la décision des investigateurs ou de la mienne, j'accepte que ma rétribution soit calculée au prorata des phases effectuées.

J'accepte de me soumettre aux exigences signalées dans le protocole, à savoir:

- m'abstenir de toute occupation pouvant remettre en cause ma participation à l'étude
- me présenter autant de fois que nécessaire (en plus des visites médicales) à l'endroit de l'étude selon l'horaire qui m'est communiqué.

Je m'engage à signaler tout écart de ma part aux prescriptions des investigateurs, de même que tout symptôme inattendu ou inhabituel.

J'ai reçu une copie de cette feuille de consentement et j'ai pu bénéficier d'un temps de réflexion suffisant pour prendre ma décision.

Lausanne, le ___________________________

Le volontaire :

L'investigateur :

Annexe 5. Formulaire pour la visite d'inclusion

Laboratoire Suisse d'Analyse du Dopage
Institut Universitaire de Médecine Légale
Chemin des Croisettes 22
1066 Epalinges

Lausanne, le 23 août 2013

Étude pharmacocinétique du métabolisme de la nicotine
après consommation de tabac fumé et non-fumé.

Formulaire pour la visite d'inclusion

Sujet n° ___ Date: ___/___/___

VISITE D'INCLUSION

Date:

Investigateur :………………………………………………………………………

Expliquer l'étude au sujet et récupérer le formulaire de consentement éclairé reçu lors de la séance d'information (le lieu, la date et la signature doivent être indiqué par le volontaire)

Nom, prénom du sujet :

Date de naissance :

Taille : **Poids :** **IMC :**

	Critères d'Inclusion	oui	non
1.	Le volontaire a entre 20 et 30 ans ?	☐	☐
2.	Le volontaire est en bonne santé ?	☐	☐
3.	Le volontaire pratique une activité physique régulière ? (pratique sportive ≥ 1x par semaine)	☐	☐
4.	Le volontaire est un fumeur occasionnel ? (1 ≤ x ≤ 3x par semaine)	☐	☐
5.	Le volontaire possède un IMC compris entre 18 et 30 ?	☐	☐
6.	Est-ce que le sujet a donné son consentement éclairé par écrit ?	☐	☐

	Critères d'Exclusion	oui	non
1.	Le volontaire consomme-t-il régulièrement des produits du tabac ? (≥ 4x par semaine)	☐	☐
2.	Le volontaire consomme-t-il des substituts nicotiniques (patch, gomme, pastille, inhaleur,...)?	☐	☐
3.	Le volontaire consomme régulièrement des drogues, de l'alcool ou des médicaments ?	☐	☐
4.	Le volontaire souffre-t-il d'ulcères gastroduodénals, de colites ulcéreuses ou de la maladie de Crohn ?	☐	☐
5.	Le volontaire souffre-t-il de maladies cardiovasculaires (arythmie, insuffisance cardiaque manifeste ou latente, hypertension artérielle) ?	☐	☐
6.	Le volontaire souffre-t-il de maladies pulmonaires (broncho-pneumopathie chronique obstructive, asthme) ?	☐	☐

7.	Le volontaire souffre-t-il de maladies néoplastiques (cancer) ?	☐	☐
8.	Le volontaire souffre-t-il de maladies inféctieuses ?	☐	☐
9.	Le volontaire souffre-t-il d'insuffisance rénale ?	☐	☐
10.	Le volontaire souffre-t-il de troubles hépatiques ?	☐	☐
11.	Le volontaire souffre-t-il d'allergies ?	☐	☐
12.	Le volontaire souffre-t-il de migraines ?	☐	☐
13.	Le volontaire souffre-t-il d'épilepsie ?	☐	☐
14.	Le volontaire souffre-t-il d'un ulcère gastroduodénal, d'insuffisance cardiaque, hépatique ou rénale, d'hypertension, de migraine, d'asthme ou d'épilepsie ?	☐	☐

Inclusion possible du sujet dans l'étude:

☐ Oui ☐ Non

Remarques :

DONNÉES PERSONNELLES

Adresse de contact :

Adresse de courrier électronique :

N° de téléphone :

N° de téléphone d'une personne à contacter en cas d'urgence :

Annexe 6. Cahier d'observations

Laboratoire Suisse d'Analyse du Dopage
Institut Universitaire de Médecine Légale
Chemin des Croisettes 22
1066 Epalinges

Lausanne, le 23 août 2013

Étude pharmacocinétique du métabolisme de la nicotine
après consommation de tabac fumé et non-fumé.

Cahier d'observations

Sujet n° ___ Date: __/__/__

CAHIER D'OBSERVATIONS DE L'ETUDE CLINIQUE

DATE	HEURE	TYPE D'INTERVENTION	VISA

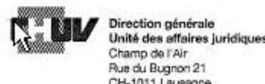
DATE	HEURE	TYPE D'INTERVENTION	VISA

DATE	HEURE	TYPE D'INTERVENTION	VISA

SUIVI DES PRODUITS DU TRAITEMENT

DATE	NOM	DOSAGE	N° DE LOT	DATE D'EXP.	REMARQUE	VISA

Annexe 7. Contrat d'assurance responsabilité civile



Direction générale
Unité des affaires juridiques
Champ de l'Air
Rue du Bugnon 21
CH-1011 Lausanne

M. Martial Saugy
Directeur du Laboratoire suisse d'analyse du dopage - LAD
Chemin des Croisettes 22
1066 Epalinges

Alberto CRESPO, Juriste
Chef de l'Unité des affaires juridiques

Tél: 021 314 18 13
Fax: 021 314 18 18

Alberto.Crespo@chuv.ch
www.chuv.ch

n/réf : er

Lausanne, le 12 octobre 2011

ATTESTATION - Essais cliniques
Autoassurance en responsabilité civile de l'Etat de Vaud dès le 01.01.2008

Etude clinique débutant :	dès l'approbation de la Commission d'Ethique de la Recherche
Titre de l'étude :	**Etude pharmacologique du métabolisme de la nicotine après consommation de tabac fumé et non-fumé**
Lieu de l'étude :	LAD et Département universitaire de médecine communautaire, CHUV
Promoteur de l'étude :	CHUV
Investigateur responsable :	M. Martial Saugy Dr ès sc.
Co-Investigateurs :	M. François Marclay MSc, Prof. Jacques Cornuz, M. Cong Khanh Huynh Dr ès sc., CHUV

Monsieur,

A la suite de votre demande concernant la recherche clinique citée en exergue, à laquelle vous serez appelé à participer en tant qu'investigateur responsable pour le CHUV, qui est dans ce cadre également promoteur, je vous confirme les points suivants :

- Suite à la résiliation de notre assurance RC avec effet au 31 décembre 2007, les collaborateurs de notre institution appelés à participer au sein de l'institution à des essais cliniques sont couverts par les ressources de notre institution.
- A ce titre, nous répondrons des dommages subis par le sujet de recherche dans le cadre de l'étude précitée qui se déroulera dans le Laboratoire suisse d'analyse du dopage et au Département universitaire de médecine communautaire du CHUV, conformément aux articles 53 et suivants de la loi fédérale sur les médicaments et les dispositifs médicaux (Loi sur les produits thérapeutiques, LPTh) ainsi que des articles 6 et suivants de l'Ordonnance sur les essais cliniques (Oclin). Ces articles figurent en annexe à la présente, pour la bonne forme.

J'espère ainsi avoir répondu à votre attente, et me tiens à votre disposition si nécessaire.

Veuillez agréer, Monsieur, mes meilleures salutations.

Elisabeth Revaz, Juriste

Annexe mentionnée

vaud

Ordonnance sur les essais cliniques de produits thérapeutiques (OClin)
du 17 octobre 2001 (Etat le 7 septembre 2004) RS 812.214.2

Art. 7 Couverture des dommages

1. Le promoteur répond des dommages subis par un sujet de recherche dans le cadre d'un essai clinique.
2. Il doit garantir cette responsabilité. A cet effet, il peut conclure pour lui-même et pour l'investigateur une assurance couvrant leur responsabilité civile contractuelle et extra-contractuelle à l'endroit des sujets de recherche.
3. S'il a son siège à l'étranger, il doit désigner une personne en Suisse qui garantisse cette responsabilité; il doit en outre accorder au sujet de recherche un droit d'action directe contre cette personne.
4. Il peut exercer un droit de recours contre l'investigateur ou d'autres personnes qui répondent des dommages subis par le sujet de recherche.
5. Le promoteur et l'investigateur peuvent convenir d'assumer ensemble la réparation des dommages selon une clé de répartition préétablie.

(Loi sur les produits thérapeutiques, LPTh)
du 15 décembre 2000 (Etat le 20 janvier 2004) RS 812.21

Art. 54 Conditions et obligation d'annoncer

1. Pour que des essais cliniques puissent être effectués, il faut notamment:
   a. que les sujets de recherche aient donné leur consentement libre, exprès et éclairé, par écrit ou attesté par écrit, après avoir été informés notamment sur:
      1. la nature et le but de l'essai;
      2. l'ensemble des actes et des analyses impliqués;
      3. l'existence d'autres traitements que ceux prévus dans l'essai;
      4. les risques, les inconforts et les bénéfices prévisibles;
      5. leur droit à une compensation en cas de dommages imputables à l'essai;
      6. leur liberté de retirer leur consentement à tout moment sans préjudice pour leur prise en charge thérapeutique;
   b. qu'une compensation pleine et entière des dommages subis dans le cadre de l'essai soit garantie aux sujets de recherche;
   c. que la commission d'éthique compétente ait donné un avis favorable.
2. Le Conseil fédéral précise les conditions auxquelles le consentement des sujets de recherche doit être obtenu.
3. Les essais cliniques doivent avoir été annoncés préalablement à l'institut. Le Conseil fédéral précise l'obligation de les annoncer. Il peut notamment:
   a. soustraire certains essais ou les essais portant sur certains produits thérapeutiques à l'obligation d'annoncer;
   b. soumettre les essais cliniques de produits thérapeutiques vétérinaires sur les animaux à l'obligation d'annoncer.
4. L'institut peut interdire un essai ou lier son exécution à des charges et à des conditions si les exigences fixées par la présente loi ne sont pas remplies. L'institut peut en tout temps procéder à une inspection en vue de contrôler l'exécution d'un essai clinique.
5. Le Conseil fédéral peut remplacer l'obligation d'annoncer les essais par une autorisation obligatoire pour certains essais cliniques tels que les essais de thérapie génique ou les essais effectués sur des sujets de recherches visés à l'art. 556 et ne leur apportant pas de bénéfice direct.
6. L'arrêt ou la fin d'un essai clinique doit être annoncé à l'institut.
7. Le Conseil fédéral peut arrêter des dispositions relatives à la publication des essais cliniques annoncés et autorisés ainsi qu'à leur arrêt ou à leur fin.

Annexe 8. Information sur les composés d'intérêt

Information sur les composés d'intérêt

Métabolisme et élimination

Le métabolisme de la nicotine comprend des phases I et II. Lors de la phase I, la nicotine est métabolisée à 70-80% en cotinine par C-oxydation et dans une moindre mesure en nicotine-*N'*-oxyde par N-oxydation. La cotinine est ensuite métabolisée en *trans*-3-hydroxycotinine par hydroxylation, en cotinine-N-oxyde par N-oxydation et en d'autres métabolites mineurs. Lors de la phase II, la nicotine, la cotinine et la *trans*-3-hydroxycotinine sont métabolisées sous forme de glucuro-conjugués.
Lors de la cessation de l'exposition, les concentrations urinaires de nicotine et cotinine diminuent avec une demi-vie d'élimination apparente d'environ 2h et 18-20h, respectivement. L'élimination est complète en l'espace de 24h et 72h, respectivement.

De même, le tabac contient d'autres alcaloïdes mineurs, en particulier l'anatabine, l'anabasine et la nornicotine. Ceux-ci sont majoritairement excrétés sous forme libre dans l'urine bien que leur métabolisme de phase II comprenne également une glucuro-conjugaison.
Suite à la cessation de l'exposition, les concentrations urinaires d'anatabine, anabasine et nornicotine diminuent avec une demi-vie d'élimination apparente d'environ 10h, 16h et 12h, respectivement. Leur élimination est complète en l'espace de 48h environ.

De plus, il existe quatre nitrosamines spécifiques du tabac, dont la plus abondante est la 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Celle-ci est métabolisée en (methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) par l'action conjointe d'une réductase carbonyle et d'enzymes. La NNAL est ensuite métabolisée sous forme de glucuro-conjugué.
Après cessation de l'exposition, les concentrations urinaires de NNAL diminuent avec une demi-vie d'élimination apparente d'environ 10 jours. Concernant l'élimination complète, les études relevées dans la littérature ne fournissent que très peu d'information à ce sujet. Toutefois, l'élimination complète peut être estimée à environ 26 et 45 jours selon que le tabac soit fumé ou consommé sous forme de snus.

Enfin, des hydrocarbones aromatiques polycycliques (PAH), et principalement du pyrène, se retrouvent couramment dans la fumée de cigarette. Son métabolisme résulte en la formation de 1-hydroxypyrène (1-OH-Py) dans sa phase I, puis de son métabolite glucuro-conjugué de phase II. Cette dernière forme représente environ 80% du métabolisme du pyrène dans l'urine.

Suite à la cessation de l'exposition, les concentrations urinaires de 1-OH-Py et 1-OH-Py-Gluc diminuent avec une demi-vie d'élimination apparente d'environ 18h. Concernant l'élimination complète, les études relevées dans la littérature ne fournissent que des informations incomplètes à ce sujet. Toutefois, l'élimination complète peut être estimée à environ 48h.
D'autres PAH tels le 1-naphthol, le 2-naphthol et le 1-hydroxyfluorène se retrouvent également en concentrations significatives dans l'urine lors du métabolisme de phase I des produits de combustion. Leur élimination complète peut être elle-aussi estimée à environ 48h.

V.4. Sociological study plan and WADA application form

Pièces obligatoires du formulaire de demande de bourse pour la recherche ciblée en sciences sociales
Agence Mondiale antidopage (AMA) – mars 2011

Effet des clubs et des pays sur la consommation de Snus en hockey sur glace : le cas de la Suisse et de la France.

Projet présenté sous la direction de Fabien Ohl – Université de Lausanne

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1. PLAN DU PROJET

1. 1. La consommation de Snus comme analyseur des effets du collectif sur les consommations de produits

La consommation de tabac non fumé semble se diffuser dans plusieurs sports en Europe (Bujon, 2008). Cette diffusion plus particulièrement marquée en hockey sur glace où un joueur sur deux testés aux championnats du monde de hockey en consomment en quantité importante en tant que stimulant (Marclay, Saugy, 2010). Plusieurs travaux (Lentillon-Kaestner, Becholey, Romand, Ohl, 2009) indiquent que cette diffusion est également importante en hockey sur glace en Suisse romande (Snus essentiellement, c'est pourquoi nous parlerons surtout de ce produit). Nous constatons une tendance à la normalisation de cette consommation parmi les jeunes joueurs de hockey. Cette propension à consommer du Snus est corrélée avec une disposition à consommer des produits destinés à améliorer les performances. Certes, les produits les plus forts du type stéroïdes anabolisants ou hormones de croissance ne s'affichent pas et ne semblent pas très courants chez les jeunes hockeyeurs suisses. Mais la consommation de Snus semble aller avec un déplacement de normes d'autres consommations et constituer un bon indicateur de la façon dont les normes de consommation de produits s'imposent collectivement. On observe en effet le plus souvent une tolérance des entraineurs et dirigeants à la consommation de Snus (Bujon, 2008).

Notre proposition de recherche est d'analyser la consommation de Snus afin de l'utiliser comme révélateur des processus de socialisation aux stimulants. Cette façon d'entrer dans la culture de consommation va nous permettre d'approcher de façon indirecte la consommation de produits dopants. Aborder le dopage, même qualitativement, n'est pas une chose facile. Cela nécessite du temps, beaucoup de confiance et c'est plutôt un travail qui se réalise sur plusieurs années (Brissonneau, Aubel, Ohl, 2008). Un questionnement direct sur le dopage entraine fréquemment une fin de non recevoir par les sportifs et des stratégies de minimisation des consommations en raison d'une attente de désirabilité sociale (Petróczi, Nepusz, 2011). En revanche, le Snus est un produit qui n'est pas classé comme dopant, qui ne fait pas partie des « secrets » de la pratique et qui, au contraire, semble s'exhiber comme norme virile partagée par les « vrais » joueurs de hockey. La norme semble si prégnante que les non consommateurs paraissent parfois exclus du cercle des initiés et que peu de joueurs résistent à l'emprise de ces nouvelles normes collectives.

La consommation de produits est rarement un processus individuel. Si l'on fait souvent la chasse aux tricheurs et si la stigmatisation des sportifs coupables de dopage semble dédouaner les organisations, la consommation de produits est à comprendre dans un contexte plus large que la simple moralité individuelle. Les pairs et les formes de socialisations établies dans les clubs jouent un rôle majeur dans la transmission de la culture de la consommation. Ainsi, les pratiques de dopage n'apparaissent pas soudainement, elles résultent au contraire d'un processus progressif de transformation des normes et de changement des rapports aux produits. Dans le cas du hockey, la consommation de Snus consitue déjà une conduite dopante au sens de P. Laure (2000) et participe, nous semble-t-il de ces changement de normes au cours de la carrière sportive. Car en effet, les carrières sportives ne sont pas simplement ponctuées par des séries de progrès physiques, elles présentent aussi une dimension morale qui transforme progressivement le rapport au monde et aux choses et déplace les normes de consommation de produit.

1. 2. Objectif : mieux connaître les rapports aux normes collectives de consommation

C'est parce que les déviances sont le plus souvent le résultat de processus collectifs, dans lesquels le déviant se socialise à de nouvelles normes et conforme ses pratiques à celles d'un groupe (Becker, 1985), qu'il est difficile d'obtenir des résultats dans la lutte contre le dopage si l'on n'essaie pas de modifier la culture de la déviance. On peut supposer que, comme dans d'autres cas (Waddington, 2009 ; Brissonneau, Aubel, Ohl, 2008), c'est au contact des pairs, des sportifs plus anciens et des diverses personnes en charge de l'encadrement (entraîneurs, dirigeants) que s'apprennent les savoir-faire mais aussi les normes qui les légitiment (Lentillon-Kaestner & Carstairs, 2010).
C'est pourquoi notre projet à plusieurs objectifs. Premièrement, de comprendre comment se transmet et se normalise la culture du Snus dans le hockey. Même si le Snus n'est pas un produit dopant, cet objectif est tout à fait légitime eu égard aux effets nocifs de cette consommation. Deuxièmement, il s'agit d'utiliser cette consommation comme révélateur de la constitution des normes collectives et de leur diffusion au sein du hockey. Cela nous permettra aussi d'appréhender le rapport aux pratiques de dopage chez les jeunes joueurs de hockey.
Nous proposons de comprendre comment débute la consommation de Snus. Qui initie ? Quelles sont les attitudes des dirigeants, entraineurs et pairs vis-à-vis des consommateurs et des non consommateurs ? Qui résiste à la consommation et à la pression du groupe ? Est-ce que des facteurs familiaux expliquent les normalisation et/ou résistances ? Quels sont les facteurs favorables à cette résistance aux normes ? Est-ce que les réseaux sociaux dans et hors du hockey jouent un rôle ? En quoi le Snus normalise la prise de produits ? Quels liens entre ces consommations et des normes viriles du hockey ? Est-ce que la normalisation de la consommation de Snus conduit à modifier le rapport aux normes de consommation de produits dopants ? Est-ce que les normes de production de la performance modifient les techniques d'entraînement et les normes de santé ou d'éthique ? Est-ce que certains joueurs passent de la consommation de produits autorisés, comme le Snus, à des produits non autorisés ?
A travers ces questionnements, nous souhaitons nous plonger dans la culture de ces jeunes joueurs pour mieux comprendre comment s'opèrent les influences du contexte, en particulier du groupe de pairs et des dirigeants, sur leurs façons de consommer des produits.

Notre objectif de mieux connaître les façons dont les consommations de Snus se normalisent, nous permettra de voir comment les risques sont progressivement déplacés voire ignorés. Selon les résultats, nous identifierons les principaux facteurs favorables à la consommation de Snus, mais aussi d'autres produits, afin de pouvoir suggérer des politiques de prévention. Nous nous mettrons également en relation avec l'OFSP (Office Fédéral de la Santé Publique) de façon à voir s'ils peuvent également s'impliquer dans des politiques de prévention ciblées.

1. 3. Méthode

Nous proposons de réaliser une analyse des effets des socialisations sportives sur la consommation de Snus en analysant les joueurs juniors et jeunes seniors de Suisse romande et de France voisine (nous n'indiquons pas le noms des clubs afin de garantir l'anonymat mais nous essaierons pour la Suisse de choisir des clubs identifiés comme « gros consommateurs » et d'autres qui le sont moins).

1. Le premier objectif est de savoir s'il existe des effets de club sur la consommation de Snus et plus largement le rapport aux normes collectives de consommation des produits dopants. Nous proposons de sélectionner trois clubs suisses et de procéder à des

entretiens semi-directifs auprès de jeunes joueurs (16 à 22 ans). Nous sélectionnerons des consommateurs en focalisant sur leur carrière de consommateur de Snus et en tentant de cerner les autres consommations de produits et les dispositions à l'égard des produits dopants. Nous veillerons à sélectionner des non consommateurs qui, bien que probablement plus rares, sont intéressants à comprendre. Ils peuvent nous aider à mieux identifier les facteurs favorables à une résistance à l'égard des normes collectives de consommation.

2. Le deuxième objectif est de comprendre si l'encadrement influence la culture de la consommation. En incitant, en partageant, en étant indifférent ou en se mobilisant afin de questionner les normes dominantes, l'encadrement peut jouer un rôle significatif dans la consommation. Des entraineurs et des dirigeants des clubs seront interrogés dans chaque club. Cela nous permettra plus largement d'aborder le rôle de l'encadrement et du suivi médical.
3. Le troisième objectif est de voir si des effets contextuels existent. Le hockey en Suisse est une pratique médiatisée qui attire des spectateurs et peut permettre une professionnalisation. Le hockey peut même contester la suprématie du football dans le champ sportif suisse. L'idée serait donc d'avoir un club témoin en France de façon à voir si le contexte institutionnel, économique et médiatique a une influence sur les consommations de Snus et plus largement le rapport aux produits.

L'échantillon de base que nous proposons est le suivant :

	Consommateurs	Non consommateurs	Entraineurs-dirigeants-Medecins
Club Suisse 1	10	5	2 ou 3
Club Suisse 2	10	5	2 ou 3
Club Suisse 3	3	2	2 ou 3
Club français 1	3	2	2
Total : 50	26	14	10

Il est à noter que si nous avons de bonnes chances de pouvoir accéder à certains clubs par le biais de réseaux de relation, il n'est pas exclu de rencontrer des refus de collaboration parmi les joueurs et les dirigeants. Si c'est le cas nous contacterions d'autres clubs et d'autres personnes pour compléter l'échantillon. Par ailleurs, l'échantillon est constitué a priori et sera adapté en fonction d'un principe de saturation des données. Si, au bout de quelques entretiens, les processus identifiés s'avèrent identiques dans l'une ou l'autre des catégories, il ne servirait à rien de poursuivre la collecte. En revanche, s'il s'avère que pour certaines catégories les informations ne saturent pas et se révèlent intéressantes à chaque nouvel entretien, il faudrait alors augmenter le quota.

Nous préparerons un guide d'entretien organisé autour de l'articulation entre carrière sportive et carrière de consommateur de produits. Nous essaierons d'identifier les moments d'entrée en consommation, les différentes étapes de la consommation et de les mettre en relation avec les types d'engagement dans le hockey.

Il est probable que ces paramètres soient particulièrement importants à comprendre pour expliquer pourquoi certains joueurs résistent et d'autres consomment des produits. Il s'agira donc de dégager les éléments qui permettent d'expliquer cette capacité à résister à la consommation, à se laisser convaincre ou à diffuser cette pratique.

Nous prendrons particulièrement en compte le rôle des réseaux de pairs, l'implication et les attitudes des proches (parents, éducateurs, dirigeants…) et la signification que revêt l'engagement dans le hockey. Il nous semble notamment qu'un engagement exclusif dans le hockey favorise

une conformité aux normes dominantes. Enfin, nous serons très attentifs aux façons dont se transforment les définitions de la santé et les perceptions du risque.

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2. BUDGET DETAILLE

Équipements et fournitures

- 2 enregistreurs numériques	$	1'180.--
- Documentation	$	500. --
- Impression brochure de prévention	$	2000.--
		Tot. 3'680.--

Assistants de recherche :

- Entretiens : estimation du temps de travail minimal 50 x 22 h = 1'100 h

Détail par entretien

- Recherche d'information 2 h
- Prise de contact 4 h
- Entretien (avec déplacement) 4 h
- Retranscription 10 h
- Analyse de contenu 2 h

Remarque : le temps nécessaire à la pénétration des réseaux est difficile à estimer précisément mais il faut compter de nombreuses heures pour les déplacements, la fréquentation des compétitions et des stages. Le temps des assistants est calculé a minima sans tenir compte de l'investissement des chercheurs porteurs du projet. On peut estimer le travail des chercheurs principaux à environ de 10 à 20% de leur temps et d'assistants de l'université à 10% soit une contribution de l'université de Lausanne et du GRSA d'au minimum 70'000 $.

- Diffusion des résultats, réunions, contacts		80 h
- Réalisation des brochures de vulgarisation		100 h
- Contact avec les organisations sportives		80 h
	Tot	260 h

Total assistant-étudiants pour 1360 h à 25 $/h $ *34'000.--*

Frais de déplacement :

- Déplacements pour les 50 entretiens $ *3'500.--*
  (forfait moyen de $ 70.-- par déplacement)

s/tot $ 41'180

Overhead de 13% de l'université (TVA inclus) et frais généraux de 6%:

- 19% $ 7'824

Total du budget $ US 49'004.—

Calendrier prévisionnel qui explique l'étalement des subventionnements:

Janvier à septembre 2012 :
contact avec les réseaux
collecte d'informations
Préparation du guide d'entretien
observations ethnographiques
entretiens de pré-enquête

Septembre 2012 à septembre 2013 :
entretiens
Début retranscription et analyse
Retour premiers entretiens

Septembre 2012 juin 2013 :
Entretiens complémentaires si nécessaire
Retranscription et analyse
Rédaction du rapport

Après le rendu du rapport et en accord avec l'AMA, diffusion des résultats selon plan prévu.

3. RENSEIGNEMENTS SUR LE CHERCHEUR PRINCIPAL ET LES PRINCIPAUX COLLABORATEURS

3. 1. Curriculum Vitae de Fabien Ohl

1. ACTIVITES PROFESSIONNELLES

- Depuis 2006 Professeur ordinaire à l'Université de Lausanne et directeur de l'Institut des Sciences du Sport de l'Université de Lausanne (ISSUL).
- 2003-2005 Professeur à l'Université Marc Bloch de Strasbourg.
- 1991-2003 Maître de conférences à l'Université Marc Bloch de Strasbourg.
- 1987-1991 Professeur agrégé à l'Université Marc Bloch de Strasbourg.

2. TITRES UNIVERSITAIRES ET QUALIFICATIONS

- 2002 Habilitation à Diriger des Recherches.
- 1991 Doctorat nouveau régime en sociologie, mention très honorable.
- 1986 D.E.A. de sociologie à l'université de Strasbourg II.
- 1985 Maîtrise en STAPS (sciences du sport).

3. TRAVAUX ET PUBLICATIONS DEPUIS 2000

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- Ohl F., Taks M. (2008), La consommation sportive, état des lieux, *Revue française de marketing*, 219, 4/5, 27-46.
- Ohl F., Taks M. (2007), Secondary socialisation and the consumption of sporting goods: cross cultural dimensions, *International Journal of Sport Management and Marketing*, 2, 1-2, 160-174.
- Lefevre B., Ohl F. (2007) Les choix des pratiques physiques et sportives des Français : omnivorité, univorité et dissonances, *Science et Motricité*, 62/3, 81-90.
- Ohl F., (2004), Goût et culture de masse : l'exemple du sport, *Sociologie et Sociétés*, XXXVI-1, 209-228.
- Aubel O., Ohl F. (2004), The Denegation of the Economy: the Example of Climbing in France, *International Review for the Sociology of Sport*, 39-2, 123-137.
- Ohl F. (2003), Les relations des consommateurs aux marques : l'exemple de l'attrait des « jeunes » pour les marques sportives, *Revue française de marketing,* 191, 2003/1, 33-48.
- Ohl F. (2003), Les objets sportifs : comment des biens banalisés peuvent constituer des référents identitaires, *Anthropologie et société*, 27-2, 167-184.
- Guilloud C., Ohl F. (2003), Femmes : les déesses du stade ? Revue *Lunes*, n°24, juillet/septembre, 70-77.
- Ohl F. (2002), La construction sociale des figures du consommateur et du client, *Sciences de la société*, 56, 24-41.
- Ohl F. (2002), Pourquoi les 12-20 ans sont-ils sensibles à l'innovation dans les produits sportifs ?, *Revue européenne de management du sport,* n°8, 39-65.
- Ohl F. (2001), Les usages sociaux des objets sportifs : le paraître « sportif » en ville, *Loisir et société/ Leisure and Society,* 24-1, 111-136.
- Ohl F. (2000), L'efficacité symbolique de « l'authenticité » sur les marchés des biens sportifs, *Revue Française de management du sport*, 1, 2000 (revue sur Internet).
- Ohl F. (2000), Les commentaires journalistiques sur le sport ont-ils un sens ?, *Recherches en communication*, 14, 185-213.
- Ohl F. (2000), Le journalisme sportif dans les quotidiens régionaux : une production sous influence, *Regards sociologiques*, 20, 109-128.
- Ohl F. (2000), Are Social Classes Still Relevant to Analyse Sports Groupings in "Postmodern" Society? An Analysis Referring to P. Bourdieu's Theory, *Scandinavian Journal of Medecine and Science in Sport*, 10-3, 146-155.

4. Mandats de recherche (depuis 2006)

En tant que requérant principal :

- 2009-2013 : FNS 100015_126658/1 « Pratique sportive et pharmacopraxis dans les salles de forme en Suisse romande ».
- 2009-2013 : OFSP (Office Fédéral de la Santé Publique), « Troubles anorexiques et pratiques sportives : le sport au service de la minceur ».
- 2007-2010 : AMA (agence mondiale antidopage), en collaboration avec les universités de Paris X Nanterre (STAPS) et Liège (Criminologie), « Socialisation secondaire et intériorisation des normes de dopage en cyclisme sur route : le cas de la Belgique, de la France et de la Suisse. », (requérant principal, direction de l'étude)
- 2007-2009 : OFSP (Office Fédéral de la Santé Publique), « Pratique sportive et conduites dopantes chez les jeunes Suisses romands, (recrutement d'un étudiant de thèse financé par le fond).

En tant que co-requérant :

- 2010-2013 AMA requérant principal Mattia Piffaretti, co-requérant F. Ohl WINDOP, Prise en charge complète de l'athlète dénoncé de dopage.
- 2009 : Baspo (office fédéral du sport de Macolin) « Substance use among young athlètes », requérant principal J.C Suris (GRSA-FBM).
- 2008 : Subside de recherche de la faculté biologie et médecine de l'Université de Lausanne pour le projet interdisciplinaire Ballabeina portant sur l'obésité enfantine (co-requérants Jardena Puder, Fabien Ohl, Pedro Marquez-Vidal, Andreas Nydegger et Patrick Bodenmann).
- 2008-2010 : FNS 100015-122381, Subside de recherche du FNS pour le projet « Agressions sportives en Suisse », requérant principal Alain Clémence.

4. RESPONSABILITÉS SCIENTIFIQUES

- RESPONSABILITÉS EDITORIALES
  - Membre de l'editorial board de *l'European Journal for Sport and Society* depuis 2010
  - Associated Editor de l'*International Review for The Sociology of Sport* depuis 2006
  - "Corresponding Editor" pour l'Europe de l'*International Review for The Sociology of Sport* entre 2004 et 2006
  - Président du comité scientifique de la *Revue Européenne de Management du Sport* depuis 2005.
  - Membre du comité de lecture de la revue *Science et motricité*.
  - Membre du comité éditorial de l'*International Review on Sport and Violence*

- RESPONSABILITÉS DANS DES ORGANISATIONS SCIENTIFIQUES
  - Vice-President de l'ISSA (Association Internationale de Sociologie du Sport) depuis 2008.
  - Executive Board member de l'ISSA (Association Internationale de Sociologie du Sport) depuis 2003. Correspondant de l'ISSA à l'ISA (Association Internationale de Sociologie).
  - Extended Board member de l'ISSA (Association Internationale de Sociologie du Sport) entre 2000 et 2003.

3. 2. Curriculum Vitae de Vanessa Lentillon-Kaestner

1. ACTIVITES PROFESSIONNELLES

2010- : Professeure formatrice, UER - Education Physique et Sportive (UER-EPS), Haute Ecole Pédagogique (HEP), 50%, Lausanne, Vaud
2006- : Chargée de cours en psychologie du sport à l'ISSUL, Faculté des Sciences Sociales et Politiques
Post doctorat (1e assistante) à l'ISSUL (80%, 50% dès Sept. 2010).
2006-2007 : Chargée de cours à l'université à l'Unité de Formation et de Recherche en Sciences et Techniques des Activités Physiques et Sportives (UFR STAPS), Université Claude Bernard Lyon 1, France et à l'ISSUL, Université de Lausanne
2004-2006 : Attaché Temporaire d'Enseignement et de Recherche (ATER) à l'Unité de Formation et de Recherche en Sciences et Techniques des Activités Physiques et Sportives (UFR-STAPS), 50%, Université Claude Bernard Lyon 1, France
Chargée de cours à l'UFR-STAPS, Université Claude Bernard Lyon 1, France
2002-2004 : Allocataire de recherche à l'UFR STAPS, Université de Lyon 1
Chargée de cours à l'UFR STAPS, Université de Lyon 1
2001-2002 : Professeur certifié en Education Physique et Sportive, Lycée Saint-Exupéry, Lyon 4e, France

2. TITRES UNIVERSITAIRES ET QUALIFICATIONS

2007 : Qualifiée Maître de Conférences en Sciences et Techniques des Activités Physiques et Sportives (STAPS) (74e section universitaire, France)
2006 : Doctorat en Sciences et Techniques des Activités Physiques et Sportives (STAPS), Université Claude Bernard – Lyon I, France
2004 : Agrégation externe en Education Physique et Sportive, France
2002 : Diplôme d'Eudes Appliquées (D.E.A.) en Sciences et Techniques des Activités Physiques et Sportives (STAPS) – Option Sport et performance, Université Claude Bernard – Lyon 1
2001 : Certificat d'Aptitude au Professorat d'Education Physique et Sportive (CAPEPS), France
Maîtrise en Sciences et Techniques des Activités Physiques et Sportives (STAPS), Option Education & Motricité, Université Claude Bernard, Lyon I

3. TRAVAUX ET PUBLICATIONS DEPUIS 2000

1. Articles publiés dans des revues à comité de lecture

Lentillon-Kaestner, V., Hagger, M., Hardcastle, S. (2011). Health and doping in elite level cycling. *Scandinavian Journal of Medicine and Science in Sports. Early view,* mars 2011 (DOI : 10.1111/j.1600-0838.2010.01281.x)

Lentillon-Kaestner, V., Ohl, F. (2010). Can we accurately measure the prevalence of doping? *Scandinavian Journal of Medicine and Science in Sports.* Early view, septembre 2010 (DOI : 10.1111/j.1600-0838 2010.01199x).

Lentillon-Kaestner, V., Carstairs, C. (2010). Doping use among elite cyclists : a qualitative psychosociological approach. *Scandinavian Journal of Medicine and Science in Sports,* **20(2),** 336-345.

Lentillon-Kaestner V. (2009). Injustices perçues en Education Physique et Sportive chez les élèves de collège et de lycée. *Les Nouveaux Cahiers de Recherche en Education*, **12(2),** 195-210.

Lentillon V., Brissonneau C. (2009). Appropriation progressive de la culture du dopage dans le cyclisme. *Déviance et Société,* **33(4),** 519-541.

Lentillon V. (2009). Les stéréotypes sexués relatifs à la pratique des activités physiques et sportives chez les adolescent(e)s français et leurs conséquences discriminatoires. *Bulletin de psychologie,* **62(1),** 15-28.

Lentillon, V. (2009). La mixité en Education Physique et Sportive : points de vue d'élèves de second degré. *Revue eJRIEPS (e Journal de la Recherche sur l'Intervention en Education Physique et Sport),* **16**, *38-54: http://www.fcomte.iufm.fr*

Lentillon-Kaestner, V. (2008). Conduites dopantes chez les jeunes cyclistes du milieu amateur au milieu professionnel. *Psychotropes, vol.* **1(14),** 41-57.

Lentillon V. (2008). Les élèves de second degré face à l'évaluation en Education Physique et Sportive. *Revue STAPS,* **79**, 49-66.

Lentillon, V. (2007). Désavantages réels et perceptions de privation chez les élèves en Education Physique Sportive. *Cahiers Internationaux en Psychologie Sociale*, **75-76,** 79-91.

Lentillon, V., Cogérino, G. & Kaestner, M. (2006). Injustice in Physical Education: gender issue and perception of deprivation in grades and teacher support. *Social Psychology of Education,* **9(3)**, 321-339.

Lentillon, V., & Cogérino, G. (2006). Perceptions chez les élèves de second degré du soutien d'enseignants plus ou moins expérimentés en EPS. *Revue STAPS,* **72,** 49-61.

Lentillon, V., & Cogérino, G. (2005). Les inégalités entre les sexes dans l'évaluation en EPS : sentiment d'injustice chez les collégiens**.** *Revue STAPS,* **68**, 79-95.

Lentillon V., & Trottin, B. (2005). Relations Educatives en Education Physique et Sportive et perceptions chez des collégiennes et collégiens Français. *Revue Education et Francophonie,* **33(1)**, 57-72: http://www.acelf.ca

Lentillon, V., & Cogérino, G. (2003). Pratiques des jeunes en milieu scolaire et inégalités sexuées. *Chronique Féminine : « Au tour des sportives »*, **83-85**, 46-49.

2. Contribution à des ouvrages

Lentillon, V., & Cogérino, G. (2006). Perceptions des élèves, des feedback émis par l'enseignant en éducation physique et sportive: effet du sexe de l'élève. In G. Carlier, D. Bouthier & G. Bui-Xuân (coord.), *Intervenir en education physique et en sport: recherches actuelles* (pp.391-396). Belgique : Presses Universitaires de Louvain.

Lentillon V. (2005). Privation, justice, inégalités: perceptions différenciées chez les filles et les garçons au niveau des notes et du soutien obtenus en EPS ? In G. Cogérino (dir.), *Filles et garçons en EPS* (pp. 183-197). Paris : Editions Revue EPS.

4. Mandats de recherche (depuis 2006)

Co-requérante à différents projets de recherche :

- 2009-2013 : OFSP (Office Fédéral de la Santé Publique), « Troubles anorexiques et pratiques sportives : le sport au service de la minceur » (rédaction du projet, direction de l'étude)
- 2007-2009 : OFSP (Office Fédéral de la Santé Publique), « Pratique sportive et conduites dopantes chez les jeunes Suisses romands » (participation à la réalisation de l'étude)
- 2010-2013 AMA requérant principal Mattia Piffaretti, co-requérante F. Ohl, V. Lentillon-Kaestner WINDOP, Prise en charge complète de l'athlète dénoncé de dopage. (participation à la rédaction du projet)

Participation à la réalisation de projets de recherche (sans être co-requérante) :

- 2007-2010 : AMA (agence mondiale antidopage), en collaboration avec les universités de Paris X Nanterre (STAPS) et Liège (Criminologie), « Socialisation secondaire et intériorisation des normes de dopage en cyclisme sur route : le cas de la Belgique, de la France et de la Suisse. » (réalisation de l'étude en Suisse romande)
- 2009 : Baspo (office fédéral du sport de Macolin) « Substance use among young athlètes », requérant principal J.C Suris (GRSA-FBM) (participation à la constitution du questionnaire, au recueil des données, et la rédaction du rapport final)

5. Responsabilités scientifiques

- Responsable de la recherche en Education Physique et Sportive (UER-EP), Haute Ecole Pédagogique, Lausanne, Vaud (HEP-VD)

- ACTIVITES DE REVIEWING

- Review « Sex Roles »
- Cahiers Internationaux de Psychologie Sociale
- International Journal of Sport Medicine

3. 3. Curriculum Vitae de Joan Carl Suris

1. PERSONAL DATA

Name	Joan-Carles Suris i Granell
Date of birth	December 31st 1957, in Barcelona (Spain)
Marital status	Married, two children aged 21 and 17
Home address	Route de la gare 24 / 1163 ETOY / SWITZERLAND
Home telephone	+4121 807 4342 / +4178 636 1183
Work Address	Bugnon 17 / 1005-LAUSANNE / SWITZERLAND
Work telephone	+4121 314 7375
Work fax	+4121 314 7244
E-mail	Joan-carles.suris@chuv.ch

2. UNIVERSITY STUDIES

1977-84	Medical School, University of Barcelona
1986-89	Residency in Pediatrics. Hospital Sant Joan de Déu, Barcelona
1990-92	Fellowship in Adolescent Health. Adolescent Health Program, University of Minnesota
1990-92	Master in Public Health. Division of Maternal and Child Health. School of Public Health, University of Minnesota
1997-2003	PhD in Public Health, *Cum Laude*, Universitat Autònoma de Barcelona

3. POSITIONS HELD

1992-97	Pediatrician of the Pediatric Emergency Ward of the Hospital Sant Joan de Déu, (Barcelona, Spain)
1992-03	Head, Adolescent Unit, Institut Universitari Dexeus (Barcelona, Spain)
1996-03	Research Director. Department of Obstetrics and Gynecology, Institut Universitari Dexeus (Barcelona, Spain)
Since 2003	Head, Groupe de Recherche sur la Santé des Adolescents, Institut Universitaire de Médecine Sociale et Préventive, University of Lausanne
Since 2003	Médecin associé, Unité multidisciplinaire pour la santé des adolescents, Department of Pediatrics, CHUV, Lausanne
Since 2007	Senior lecturer, University of Lausanne

4. RESEARCHES

1993-94	Principal investigator, Barcelona Adolescent Health Survey 1993, Catalan Society of Pediatrics
1995-96	Principal investigator, Psychosocial study of children of divorced parents, Catalan Society of Pediatrics
1998-99	Principal investigator, Barcelona Adolescent Health Survey 1999, Santiago Dexeus Foundation
2000-01	Principal investigator, Catalonia Adolescent Health Survey 2001, Catalan Secretariat of Youth
2003-04	Coordinator, Physical and sports activity among Swiss adolescents, Office Fédéral du Sport
2004-05	Principal investigator, Factors associated to alcohol misuse among adolescents in Vaud, Dîme de l'Alcool
2005	Coordinator, Factors influencing treatment adherence among HIV positive adolescents, Cohorte suisse VIH mère + enfant (MoCHiV)
2005	Principal investigator, Project Vulnerable Youth, Federal Office of Public Health
2006-07	Principal investigator, Modelisation of HPV Seroconversion in adolescent girls in Switzerland, GlaxoSmithKline
	Principal investigator, Transition from pediatric to adult health care for adolescents suffering from chronic conditions, Wyeth Foundation Switzerland

	Principal investigator, Modes of consumption of cannabis among youth aged 16-20 years in French-speaking Switzerland, Service of Public Health of the Canton de Vaud
2007-08	Principal investigator, Obesity project: the opinion of the actors, Service of Public Health of the Canton de Vaud
2008	Principal investigator: Health structures in sexual and reproductive health: where are the boys? Fonds Universitaire Maurice Chalumeau
	Principal investigator: Tobacco and cannabis use trajectories from adolescence to young adulthood. OFSP-Fonds de prévention du tabagisme
	Principal investigator: Substance use among young athletes. Office fédéral du sport/Macolin
	Co-investigator: Cohort study on substance use risk factors (C-SURF). Fonds National de la Recherche Scientifique
2009	Co-investigator: Children with special health care needs in Switzerland: prevalence, health care utilization and social determinants. Fonds National de la Recherche Scientifique
	Principal investigator: Unwanted sexual experiences among adolescents. Service of Public Health of the Canton de Vaud
2010	Principal investigator: Importance de l'exposition au tabac chez les jeunes consommateurs de cannabis: un cheval de Troie? Service of Public Health of the Canton de Vaud
	Principal investigator: Gambling among adolescents in Neuchâtel. Groupement Romand d'Etude des Addictions

5. HONORS AND AWARDS

1988	Scholarship "Josep M. Sala Ginabreda" for the project: "Study of the risk factors associated to meningitis and/or sepsis by multiresistant microorganisms in the child"
	Scholarship for a research project of the Catalan Society of Pediatrics for the project: "Review of the adolescents admitted in a P.I.C.U. 1977-1987"
1989	Scholarship "Societat Catalana de Pediatria" for a short stay abroad
	Fellowship "Fundació Caixa de Pensions" for advanced studies in the United States
1993	Accesit to the Scholarship "Antics Presidents de la Societat Catalana de Pediatria" for the project: "Health survey of adolescents in Barcelona"
1994	Award of the Asociación de Pediatría Extrahospitalaría de la Provincia de Alicante for the oral presentation: "What do adolescent think about their physician? A comparison between pediatricians and adult physicians"
1995	Semi-finalist Young Investigator Award of Excellence of the Society for Adolescent Medicine
1996	Pere Calafell Award 1996 of the Catalan Society of Pediatrics for the project: "Psychosocial study of children of divorced parents"
1997	Best presentation award: "Factors influencing the reliability of contraceptive methods among adolescents". Catalan Society of Contraception
2009	International Chapter Recognition Award 2009 of the Society for Adolescent Medicine

LIST OF PUBLICATIONS DR JOAN-CARLES SURIS (LAST 5 YEARS)

Website: http://www.researcherid.com/rid/C-5897-2009

A) Peer-reviewed articles

Surís JC, Parera N. Don't stop, don't stop: physical activity and adolescence. International Journal of Adolescent Medicine and Health 2005; 17 (1): 67-78.

Surís JC, Nebot M, Parera N. Behavior evaluation for risk taking adolescents (BERTA): an easy to use and assess instrument to detect adolescent's risky behaviors in clinic. European Journal of Pediatrics 2005; 164 (6): 371-6.

Surís JC, Parera N. Sex, drugs and chronic illness: health behaviours among chronically ill youth. European Journal of Public Health 2005; 15: 484-8..

Surís JC, Parera N. Being old-for-grade and drug use: not such a clear connection. Acta Paediatrica 2005; 94: 1807-14.

Surís JC, Michaud PA, Chossis I, Jeannin A. Towards a sedentary society: trends in adolescent sport practice in switzerlad (1993-2002). J Adol Health 2006; 39 (1): 132-4.

Michaud PA, **Surís JC**, Deppen A. Gender-related psychological and behavioural correlates of pubertal timing in a national sample of Swiss adolescents. Mol Cell Endocrinol. 2006;254-255:172-8.

Surís JC, Jeannin A, Chossis I, Michaud PA. Piercing among adolescents: Body art as risk marker: A population-based survey. J Fam Pract. 2007 Feb;56(2):126-30.

Zufferey A, Michaud PA, Jeannin A, Berchtold A, Chossis I, van Melle G, **Surís JC**. Cumulative risk factors for adolescent alcohol misuse and its perceived consequences among 16 to 20 year old adolescents in Switzerland. Prev Med. 2007;45(2-3):233-9.

Surís JC, Akre C, Berchtold A, Jeannin A, Michaud PA. Some go without a cigarette: characteristics of cannabis users who have never smoked tobacco. Arch Pediatr Adolesc Med. 2007 Nov;161(11):1042-7.

Chariatte V, Michaud PA, Berchtold A, Akré C, **Surís JC**. Missed appointments in an adolescent outpatient clinic: descriptive analyses of consultations over 8 years. Swiss Med Wkly. 2007 Dec 1;137(47-48):677-81.

Chariatte V, Berchtold A, Akré C, Michaud PA, **Surís JC**. Missed appointments in an outpatient clinic for adolescents, an approach to predict the risk of missing. J Adolesc Health. 2008 Jul;43(1):38-45.

Thurnherr J, Berchtold A, Michaud PA, Akre C, **Surís JC**. Violent adolescents and their educational environment: a multilevel analysis. J Dev Behav Pediatr. 2008 Oct;29(5):351-9.

Haller DM, Michaud PA, **Surís JC**, Jeannin A, Narring F. Opportunities for prevention in primary care in a country with universal insurance coverage. J Adolesc Health. 2008 Nov;43(5):517-9.

Surís JC, Michaud PA, Akre C, Sawyer SM. Health risk behaviors in adolescents with chronic conditions. Pediatrics. 2008 Nov;122(5):e1113-8.

Thurnherr J, Michaud PA, Berchtold A, Akré C, **Surís JC**. Youths carrying a weapon or using a weapon in a fight: what makes the difference? Health Educ Res. 2009;24(2):270-9.

Dominé F, Berchtold A, Akré C, Michaud PA, **Surís JC**. Disordered Eating Behaviors: What About Boys? J Adolesc Health 2009; 44(2): 111-7.

Michaud PA, **Surís JC**, Thomas R, Kahlert C, Rudin C, Cheseaux JJ, and Swiss Mother and Child HIV Cohort Study (MoCHiV). To Say or Not to Say: A Qualitative Study on the Disclosure of Their Condition by Human Immunodeficiency Virus–Positive Adolescents. J Adolesc Health 2009; 44(4): 356-62.

Pittet A, Berchtold A, Akré C, Michaud PA, **Surís JC**. Sport practice among adolescents with chronic health conditions. Archives of Pediatrics and Adolescent Medicine 2009; 163(6):565-71.

Mauerhofer A, Berchtold A, Michaud PA, **Surís JC.** The GPs role in the detection of the psychological problems of young people: a population-based study. British Journal of General Practice 2009; 59: e308-e314.

Akre C, Berchtold A, Michaud PA, **Surís JC**. Cannabis and tobacco use: Where are the boundaries? Health Education Research 2009 Jun 10. [Epub ahead of print].

Mauerhofer A, Akre C, Michaud PA, **Surís JC**. La médecine ambulatoire youth-friendly. Archives de Pédiatrie 2009; 16(8): 1151-1157.

Pittet I, Berchtold A, Akre C, Michaud PA, **Surís JC**. Are adolescents with chronic conditions particularly at risk of bullying? Arch Dis Child 2009 Mar 22.

Surís JC, Akre C, Rutishauser C. How adult specialists deal with the principles of a successful transition. Journal of Adolescent Health 2009; 45(6): 551-5.

Surís JC, Akre C, Berchtold A, Bélanger RE, Michaud PA. Chonically connected? Internet use among adolescents with chronic conditions. Journal of Adolescent Health 2010;46 (2): 200-2.

Berchtold A, Michaud PA, Nardelli-Haefliger D, **Surís JC**. Vaccination against human papillomavirus in Switzerland: simulation of the impact on infection rates. International Journal of Public Health 2010; 55 (1): 25-34.

Michaud PA, **Surís JC**, Thomas R, Gnehm HP, Cheseaux JJ. Coping with an HIV infection: A multicenter qualitative survey on HIV positive adolescents' perceptions of their disease, therapeutic adherence and treatment. Swiss Medical Weekly 2010; 140(17-18):247-53.

Akré C, Michaus PA. **Surís JC**. 'I'll look it up on the web first': Barriers and overcoming barriers to consult for sexual dysfunction among young men. Swiss Medical Weekly 2010; 140(23-24):348-53

Bucher della Torre S, Akre C, **Surís JC**. Obesity prevention opinions of school stakeholders: a qualitative study. Journal of School Health 2010;80(5):233-9.

Berchtold A, Jeannin A, Akré C, Michaud PA, **Surís JC**. First use of multiple substances: Identification of meaningful patterns. Journal of Substance Use 2010, 15(2): 118–130

Meier M, Berchtold A, Akré C, Michaud PA, **Surís JC**. Who eats healthily? A population-based study among young Swiss residents. Public Health Nutr. 2010 May 4:1-8. [Epub ahead of print].

Berchtold A, Akré C, Jeannin A, Michaud PA, **Surís JC**. First consumption ever of multiple substances: Applying an expert-based taxonomy to a Swiss national sample of adolescents. Addict Behav. 2010 Sep 17. [Epub ahead of print].

Luder MT, Berchtold A, Akré C, Michaud PA, **Surís JC**. Do youths gamble? You bet! A Swiss population-based study. Swiss Med Wkly. 2010 Aug 9;140:w13074. doi: 10.4414/smw.2010.13074

Luder MT, Pittet I, Berchtold A, Akré C, Michaud PA, **Surís JC**. Associations Between Online Pornography and Sexual Behavior Among Adolescents: Myth or Reality? Arch Sex Behav. 2011 Feb 3. [Epub ahead of print]

Rutishauser C, Akré C, **Surís JC**. Transition from pediatric to adult health care: expectations of adolescents with chronic disorders and their parents. Eur J Pediatr. 2010 Dec 22. [Epub ahead of print]

Baumann P, Bélanger RE, Akré C, **Surís JC**. Increased risks of early sexual initiators: time makes a difference. Sexual Health (in press)

Bélanger RE, Akré C, Kuntsche E, Gmel G, **Surís JC**. Mixing tobacco to cannabis – its frequency and likely implications. Nicotine & Tobacco Research (in press)

B) Synthesis articles

Michaud PA, **Surís JC**. Sports activity during adolescence: a challenge for health professionals and for the society. Rev Med Suisse 2005; 1: 1835-6, 1838-9

Surís JC, Michaud PA. Médecine de l'adolescence. Consultation garcons. Revue Médicale Suisse 2007; 3 (93): 30-33.

Surís JC, Dominé F, Akré C. La transition des soins pédiatriques aux soins adultes des adolescents souffrant d'affections chroniques. Rev Med Suisse. 2008 Jun 11;4(161):1441-4.

Pittet I, **Surís JC**. Bullying – De quoi s'agit-il? Re Med Suisse 2009; 5: 53-8

C) Monographs

Surís JC, Michaud PA, Jeannin A. Facteurs liés au mésusage d'alcool chez les adolescents vaudois de 16 à 20 ans. Rapport pour la Dîme de l'Alcool (2005) (Monograph).

Michaud PA, **Surís JC**, Jeannin A. Physical and sports activity among Swiss adolescents. Rapport pour l'Office fédéral du sport (2005) (Monograph).

Chollet A, Cornuz J, Michaud PA, **Surís JC**. Desaccoutumance au tabac chez les jeunes. Lausanne: Institut universitaire de médecine sociale et préventive; 2005 (Monograph).

Surís JC, Berchtold A, Nardelli D, Michaud PA. Modeling of HPV seroconversion in adolescent girls in Switzerland. Rapport pour la Commission fédérale de vaccination (EKIF). (Monograph)

Akre C, Michaud PA, **Surís JC**. Les modalités de consommation de cannabis chez les adolescents. Une étude qualitative. Lausanne: Institut de médecine sociale et préventive; 2008. (Monograph)

Bucher della Torre S, Akre C, Michaud PA, **Surís JC**. Prévention de l'obésité dans les écoles vaudoises: l'opinion des acteurs. Une étude qualitative. Lausanne: IUMSP; 2008. (Monograph)

Surís JC, Berchtold A, Bélanger R, Akré C. Tobacco and cannabis use trajectories from adolescence to young adulthood. Lausanne: IUMSP; 2010.

Bélanger RE, Ohl F, Berchtold A, Lentillon-Kaestner V, **Surís JC**. Substance use among sportive adolescents in the French-speaking part of Switzerland. Lausanne: IUMSP; 2010.

Bélanger RE, Marclay F, Berchtold A, Akré C, Saugy M, Michaud PA, Cornuz J, **Surís JC**. Importance de l'exposition au tabac chez les jeunes consommateurs de cannabis: un cheval de Troie? Lausanne: IUMSP; 2010. (Raions de Santé, 172)

D) Contributions

Surís JC (Coordinator). Actuacions preventives a l'adolescència. Guia per a l'atenció primària de salut. Generalitat de Catalunya, Barcelona, 2005 (Book, 174 pages).

Michaud PA, Chossis I, **Surís J**C. Health-related behavior: current situation, trends and prevention. In: Jackson S and Goossens L (Eds): Handbook of adolescent development. New York: Psychology Press, 2006. (Chapter)

Surís JC. Jeunes vulnerable en Suisse: revue de la literature et analyse secondaire des données SMASH. Dans: Prévention auprès des jeunes vulnérables. Berne: Office federal de la santé publique, 2006. (Chapter)

Michaud PA, **Surís JC**, Viner R. The adolescent with a chronic condition. Epidemiology, developmental issues and health care provision. Geneva: World Health Organization; 2007

3. 4. Curriculum Vitae de Martial Saugy

Biography :

Name : Saugy
First name : Martial
Born : 28.12.54 in Payerne (Switzerland)
Family : Married to Rita Saugy-Renevey
3 children Aurélia (1980), Jonas (1984) et Jérémy (1987)

Academic study :

1973-1979 : Licence in biology, University of Lausanne
1980-1981 : Degree in science pedagogy
1981-1986 : PhD in Plant Physiology, University of Lausanne
1986-1987 : Post-doctoral fellowship in Biochemistry, McGill University in Montreal, Canada

Professional activities:

1981-1986 : Teaching assistant in Biology, University of Lausanne
1986-1987 : Research Associate in Biochemistry, McGill University
1988-1990 : Biochemist, Laboratory of Analytical Toxicology, Legal Medecine Institute, University of Lausanne
1990-2000 : Deputy Scientific Director, Swiss Laboratory for Doping Analyses, University of Lausanne
2001- : Director of the Swiss Laboratory for Doping Analyses, University of Lausanne

4. TEACHING ACTIVITIES:

1980-1981: Teaching in Biology and Physics in secondary school.
1981-1986: Supervision and teaching in Departments of Biology and Pharmacy, University of Lausanne
1990- : Several third cycle courses and seminars on "Doping, Analytical and Research Approaches" to sport physicians, sport students and students in biology and medicine.
2006- Privat-Docent at University of Lausanne

Involvement with committees and commissions of national and international bodies:

- Scientific advisor to the Sporting Safety & Condition Commission of the UCI.
- Member of the FIFA anti-doping commission.
- Member of the List Committee of WADA
- Member of the Anti-doping Panel of UEFA
- Member of Medical and Anti-Doping commission of IAAF.

Involvement as expert in international Sports events (selection of events):

- **Sydney Olympics 2000**: Scientific delegate for cycling
- **FIFA World Cup 2002**: Laboratory expert for FIFA in Tokyo
- **UEFA Euro 2004**: Laboratory expert for UEFA in Lisbon
- **IAAF World Championships Helsinki 2005: Laboratory expert and blood tests supervision.**
- **Torino Olympics 2006**: Mandate for Blood doping analyses
- **IAAF World Championships Osaka 2007:** Laboratory expert and blood tests supervision in Osaka and Tokyo.
- **UEFA Euro 2008 Swiss-Austria**: Lab director for the event
- **IAAF World Championships Berlin 2009:** Laboratory expert in Berlin, Cologne and Dresden.
- **Vancouver 2010:** Assistant-Director of the Anti-doping Laboratory (Director: Christiane Ayotte)

Present research projects, main topics:

Human growth hormone and sport.
In charge of implementation of GH test in anti-doping field
Erythropoietin and sport
In charge of several projects supported by WADA. Federal office for sports for the detection of abuse of Rh-EPO in sport.
Nandrolone and precursors
Project supported by the FIFA medical commission and WADA to solve the major problem of the use of nor-steroids in sport.
Testosterone and endogenous steroid profile
Project for the development of a precise methodology (Isotope Ratio Mass Spectrometry) for the detection of new steroid drugs. Supported by Swiss Federal Office for Sport and FIFA
Biological Passport of the athlete
Development of longitudinal follow up of the athlete through regular blood testing. Establishment of individual reference ranges for different types of hormones and biochemical parameters or markers.

Extra-professional activities :

Sport practice : Marathon
Member of Panathlon Club in Lausanne

Member of Sports Manager Swiss Association

Invited conferences

2002

- Nandrolone and Football. Origin of low concentrations of norandrosterone in player's urine.
  100 years of Real Madrid. , Madrid, August 2002
- Detection of urinary recombinant erythropoietin
  Conférence at WADA-USADA meeting on doping and oxygen transport.
  Atlanta, September 2002.
- Développement et stratégie d'analyse des nouveaux produits dopants.
  Conférence à l'assemblée générale de la société suisse de chimie clinique.
  Zurich, October 2002.
- EPO Doping. How can we analyse it. Is urine better than blood ?
  FMSI, Bologna, november 2002
- Les analyses de dopage dans le sport. Quoi de neuf ?
  Conférence à l'institut de Médecine Légale de l'Université,
  Genève, december 2002

2003

- Cas limites dans les contrôles anti-dopage. Effet sur la performance et la santé des joueurs
  Conférence de la commission médicale de l'Union Européenne de Football Association.
  Nyon, Mai 2003
- La testostérone ou la Nandrolone. Des incertitudes des scientifiques aux décisions des tribunaux.
  Conférence au symposium de l'association suisse des managers du sport
  Musée Olympique, Lausanne, October 2003
- Detection of nandrolone and EPO
  Teams sport medical commissions meeting, FIFA.
  Zurich, November 2003.
- EPO Doping and haematological tests in sport
  Conférence au symposium scientifique de Swiss Olympic sur le dopage génétique
  Berne, November 2003

2004

- Activité du Laboratoire Suisse d'Analyse du Dopage : perspectives et développement *Commission du Conseil de l'Europe pour la surveillance de l'application de la Convention Européenne contre le dopage,*

Palais Fédéral, Berne, April 2004
- déjeuner thématique sur les enjeux du dopage, *séminaire sport et finance 2004*
  Genève, April 2004
- Le Laboratoire Suisse d'Analyse du Dopage, présentation des activités
  BASPO, Macolin, Mai 2004
- Le Laboratoire Suisse d'Analyse du Dopage, présentation des activités
  Tribunal Arbitral du Sport,Lausanne, juin 2004
- New strategies and emerging methods in doping analyses
  Ecole doctorale de pharmacie (Université de Genève),
  Zermatt, Septembre 2004
- Techniques d'analyses antidopage et droit du sport
  Journée Suisse du droit du sport,
  Macolin, Novembre 2004
- Haematological tests to detect blood manipulation in sports
  Meeting of National Anti-Doping Agencies (ANADO),
  Bern, November 2004
- Nouvelles stratégies et méthodes émergentes dans les analyses de dopage
  Douzièmes Journées franco-suisses de pharmacie hospitalière,
  Interlaken, novembre 2004

2005

- Dopage : Etat de la question depuis le laboratoire,
  Conférence publique de l'office fédéral de sport à Ténéro (Tessin)
  Ténéro, mars 2005
- Le sang du Dopage : La lutte anti-dopage, moyens actuels et perspectives
  Conférence publique organisée par la facutlé des SSP de l'UNIL dans le cadre de l'année internationale pour le sport
  Collège propédeutique, Lausanne, April 2005
- La lutte anti-dopage : moyens actuels et perspectives
  Société fribourgeoise de Médecine
  Hôpital de Riaz (Bulle) , April 2005
- Gagner sans tricher, conférence et table ronde publique, dans le cadre d'un symposium « sport, média, éthique »
  Commission suisse pour l'Unesco, Musée Olympique, Lausanne, Mai 2005
- First case of homologous transfusion in 2004
  3rd asian conference of science and football medicine
  Muscat, Oman, May 2005
- Sur le front du dopage : la lutte anti-dopage est-elle nécessaire dans le sport amateur.
  Conférence publique dans le cadre du symposium « à vos casques » organisé par la Clinique romande de réadaptation
  Sion, June 2005
- Analyse du Dopage: Des technologies de pointe pour une certaine éthique dans le sport ou mythe de Sisyphe ?
  αCTA, 10èmes journées scientifiques,
  Les Diablerets, septembre 2005

2006

- Les tests sanguins et les analyses de l'EPO dans l'urine
  Conférence sur invitation organisée par l'Association coureurs professionnels,
  Liège, Belgique , avril 2006
- Les possibilités et les limites de la lutte anti-dopage
  Conférence sur invitation de la société genevoise de Chimie
  Genève, Avril 2006
- Nouvelles stratégies dans le dopage et moyens de dépistage
  Dans le cadre des conférences du Département de médecine interne du CHUV
  Lausanne, 2006
- La lutte anti-dopage : Nouvelles stratégies pour faire face aux enjeux de Pékin 2008
  Conférence à l'Université Populaire de la Broye
  Payerne, 2006
- La lutte anti-dopage : Nouvelles stratégies pour faire face aux enjeux de Pékin 2008
  Conférence workshop Unilabs, Porte des Iris
  Vuillerens, 2006

- Un petit thé de coca à la mi-temps
  Conférence au café scientifique organisé dans le cadre de la Coupe du Monde 2006
  UNIL, June 2006
- Laboratory drug testing procedures : Future trends
  Conférence dans le cadre de la formation des contrôleurs UEFA
  Nyon, juin 2006.
- Testosterone and blood issues
  Sports medical Meeting FIFA,
  Brugge, Belgique, oct. 2006
- La lutte anti-dopage et ses nouvelles stratégies face aux nouveaux enjeux
  Conférence sur invitation Fédération Suisse d'Athlétisme
  Fribourg, Novembre 2006

2007

- Dopage : Information, prévention
  Conférence du service de l'éducation physique et des sports du canton de Vaud
  Gymnase Auguste Piccard, Lausanne, February 2007
- La lutte anti-Dopage : Nouvelles stratégies
  Colloque hebdomadaire de l'Hôpital Riviera-Chablais
  Montreux, March 2007
- The scientific Principles of Hematological Passport
  WADA group for blood tests
  Lausanne, June 2007
- Contrôles de dopage dans le sport : A la recherche de l'urine idéale,
  Conférence pour formation des contrôleurs anti-dopage de Swiss Olympic,
  Macolin, décembre 2007

2008

- Lutte contre le dopage : Etat des lieux et Avenir
  Conférence au centre mondial du cyclisme à Aigle devant les Conseillers d'Etat au sport des cantons romands.
  Aigle, February 2008
- Some Principles for the Hematological Passport,
  Meeting Association of national anti-doping agencies.
  Lausanne, April 2008
- Ethique dans le football,
  Symposium AISTS au Musée Olympique
  Lausanne, avril 2008
- The Lab behind the doping tests : Current Challenges and Future Directions
  Conference at Department of Clinical Research, University Bern
  Hôpital de l'Isle, Bern, June 2008
- Le futur dans la lutte anti-dopage
  Séminaire Centre médical de Swiss Olympic , Vidy-med
  Lausanne, September 2008

2009

- Lutte anti-dopage : Est-ce que les scientifiques resteront toujours derrière.
  Conférence au département de Médecine interne del'Hòpital Pourtalès
  Neuchâtel, 13.01.2009
- Dopage : quel avenir pour les tricheurs : Conférence – débat
  Société suisse des officiers
  Sauvabelin – Lausanne 23 April 2009
- Pitfalls in doping analysis : Why are laboratory controls not fully infallible?
  Conference at Sportmed (société Suisse des médecins du sport)
  Berne, 7 Mai 2010
- *Dopage : Quel avenir pour les tricheurs*
  Conférence sur invitation de la ligue de football de l'Ain.
  Divonne-les-Bains, 18 Mai 2009
- Dans l'intimité du couple dopeur, dopé
  Café actu, Conférence débat
  Lausanne, 26 Mai 2009

- Analytical procedures in anti-doping tests
Swiss Biotech Academy event,
Centre Génral Guisan, Pully, 24 September 2009
- IAAF blood testing program
Medical commission of IAAF
Monaco, 16 October 2009
- *Back on track with Blood*
FIFA International Medical Conference
Zurich 17 October 2009
- *The Athlete Passport: Hematological Module*
WADA-JADA Blood Doping Symposium
Tokyo , 6-8 November 2009
- *Dopage: Quel avenir pour les tricheurs?*
Conférence Panathlon d'Yverdon pour les sportifs du Nord Vaudois
Yverdon, 17 November 2009
- *Le Passeport Biologique*
1ères Journées Scientifiques de l'ANAD
Tunis, 24 November 2009
- *Hormone de croissance et Dopage*
1ères Journées Scientifiques de l'ANAD
Tunis, 24 November 2009

2010

- Recherche au Laboratoire Suisse d'Analyse du Dopage : Les défis d'aujourd'hui et de demain
Press Conference of Anti-Doping Swiss au Musée Olympique
Lausanne, 19 April 2010
- Le Laboratoire suisse d'analyse du dopage : mandats et organisation pendant les grandes épreuves sportives.
5 à 7 de la Finance, Banque Cantonale Vaudoise
Musée Olympique, Lausanne, 21 April 2010
- Dopage : Fléau du Sport
Conférence suisse des Procureurs 2010
Lausanne, 5 Mai 2010
- Anti-doping in Ice Hockey
Sports Medicine KHL Seminar
Leukerbad, 6 Mai 2010
- Tous Dopés : Quand performances riment avec substances
  Forum Drogues et autres substances – Ligue valaisanne conter la toxicomanie
  Sion 2 June 2010
- *Le Passeport Biologique : Une nouvelle approche dans la lutte anti-dopage*
Journée Romande de Médecine et de Sciences Forensiques
Université de Lausanne, Dorigny, 7 June 2010

Other Conferences (Abstracts

2003

- C13-labelled nandrolone excretion in trained athletes
  Baume Norbert, Schweizer Carine, Avois Lidia, Cardis Christine, Mangin Patrice, Saugy Martial
  21st Cologne Workshop on Dope Analysis, mars 2003
- Development and validation of a capillary electrophoresis method for the determination of ephedrine and related compounds in urine without extraction
  Avois Lidia, Saugy Martial, Mangin Patrice
  Congrès SSMS, 23-24 octobre 2003, Schaffhausen21st Cologne Workshop on Dope Analysis, 16-21 mars
- C13-labelled nandrolone excretion in trained athletes : effect of exercise
  Baume Norbert, Schweizer Carine, Avois Lidia, Cardis Christine, Mangin Patrice, Saugy Martial
  Congrès SSMS, 23-24 octobre 2003,
- High amount of salbutamol : a case report
  Schweizer Carine, Mangin Patrice, Saugy Martial
  Congrès SSMS, 23-24 octobre 2003, Schaffhausen
  21st Cologne Workshop on Dope Analysis, mars 2003

2004

- Longitudinal study « Doping Free Sport » : first results on blood analysis
  Bader S., Robinson N., Saugy M., Clénin G., Kamber M., Marti B.
  Congrés de la Société Suisse de Médecine du Sport, octobre 2004, Locarno
- Testosterone excretion study monitored by gas chromatography / combustion / isotope ratio mass spectrometry
  Maître A., Schweizer C., Saudan C., Mangin P., Saugy M
  CHUV research day, January 24th, 2004, Lausanne
- Mass spectrometry : a new technique to tackle abuse of erythropoietin (EPO) in sport?
  Saudan C., Robinson N., Mangin P., Saugy M.
  CHUV research day, January 24th, 2004, Lausanne
- ^{13}C-labelled nandrolone excretion in trained athletes during exercise
  Baume N., Avois L., Cauderay M., Dvorak J., Mangin P., Saugy M.
  American College of Sports Medicine, 51st Annual Meeting, Indianapolis, USA
- Genetic identification of urine samples used for doping control analysis
  Dimo-Simonin N, Castella V, Robinson N., Saugy M, Mangin P.
  15th International Symposium on Human Identification, Phoenix, USA
- Rapid screening of HES in urine with colorimetric detection
  Avois L., Mangin P., Saugy M.
  22nd Cologne Workshop on Dope Analysis, march 2004
- Identification of 3'hydroxystanozolol in urine by GC-MS^3 : a case report
  Avois L., Mangin P., Saugy M.
  22nd Cologne Workshop on Dope Analysis, march 2004

2005

- Homologous Blood Transfusion detection. A new world in anti-doping field: Flow cytometry. S. Giraud, N. Robinson, P. Mangin & M Saugy. *23rd Cologne Workshop on Dope Analysis, march 2005*
  - Optimization of morphine and codeine hydrolysis in urine by experimental design fro GC-MS quantification purpose. L. Avois. & M Saugy. *23rd Cologne Workshop on Dope Analysis, march 2005*
  Concentration of nandrolone metabolites in urine after the therapeutic administration of an ophtalmic solution. *23rd Cologne Workshop on Dope Analysis, march 2005*

2006

- La spectrométrie de masse dans la lutte anti-dopage, M.Saugy & C Saudan
  Congrès de la Société Française de spectrométrie de masse, Nantes, Septembre 2006
- EPO and Blood doping Detection, M.Saugy
  2006 IAAF World anti-doping Symposium , Lausanne, October 2006
- Homologous Blood transfusion proficiency tests. S Giraud, C. Georgakopoulos, J de Ceaurriz, C Ayotte, J Segura & M. Saugy
  24thCologne Workshop on Dope Analysis, Cologne, March June 2006

2007

- Détection biochimique des dopages hormonaux M. Saugy, Collège de France, 50èmes journées internationals d'endocrinologie clinique, Paris, 2007
- Synthetic glucocorticoid administration : Urinary excretion of triamcinolone acetonide. L.Avois, Bailloud Demarchelier, M. Saugy et al *25thCologne Workshop on Dope Analysis, Cologne, February 2007*
- The World Anti-doping Code: From implementation to future Challenges: International Standards for Laboratories. M Saugy, *Congrès de "European Council of Sports Sciences, Lausanne, 2007*
- EPO and Blood doping Detection, M Saugy,
  FEMEDE 07, *Congrès espagnol de médecine du sport, Séville , Oct 2007*

2008

Bailloux I, Avois L, Desmarcheliers A, Lahaussois A, Méchin N, Gremion G, Saugy M, de Ceaurriz J. Elimination of seven synthetic glucocorticosteroids in urine according to the route of administration: preliminary results. *26nd Cologne Workshop on Dope Analysis, 24-29 Février 2008, Cologne (Allemagne)*

Articles in peer-review publications :

1. **M.Saugy** and PE Pilet 1984. Endogenous indol-acetic-acid in stele and Cortex of gravistimulated roots. *Plant Science Lett,37, 93-99*
2. PE Pilet and **M. Saugy** 1985. Effect of Applied IAA on maize root growth . *Planta 164 , 254-258*
3. L. Rivier and **M. Saugy** (1986) Chemical Ionization Mass Spectrometry of Indol-3yl-Acetic Acid and Cis-Abscisic Acid : evaluation of negative ions detection and quantification of Cis-Abscisic Acid in growing maize roots. *Journal of Plant Growth Regulation, 5, 1-16*
4. P Reymond, **M Saugy** and PE Pilet 1987 Quantification of Abscissic Acid in a single maize root. *Plant Physiol 85, 8-9*
5. PE Pilet and M. Saugy 1987. Effect on Root Growth of Endogenous and Applied IAA and ABA. *Plant Physiol. 83, 33-38*
6. **M Saugy** and PE Pilet 1987 Changes of in the level of free and ester indolyl-acetic acid in growing maize roots. *Plant Physiol 85,*
7. **M Saugy**, G Mayor and PE Pilet 1989. Endogenous ABA in growing Maize roots: Light effects. *Plant Physiol. 89, 622-627*
8. **M Saugy,** V. Farkas and G. Maclachlan 1988: Phosphatases and Phosphodiesterases interfere with 1,3-beta-D-glucan synthase activity in pea epicotyl membrane preparations. *Eur. J. Biochem. 177 (1) 135-138*
9. **M Saugy**, P.Meuwly, A. Munafo and L Rivier(1991) Rapid high-performance liquid chromatographic determination with fluorescence detection of furosemide in human body fluids and its confirmation by gas chromatography-mass spectrometry. *J. Chromatography B 564, 567-578*
15. **M. Saugy**, C. Cardis, C. Schweizer, J.-L. Veuthey and L. Rivier (1996) The detection of human growth hormone doping in urine : Out of competition tests are necessary. *J. Chromatography B 687, 201-211.*
16. JR Poortmans, H. Auquier, V. Renaut, A. Durussel, **M. Saugy** and GR. Brisson.(1997) Effect of short term creatine supplementation on renal responses in men. *Eur. J. applied Physiol. & Occ. Physiol. 76 (6): 566-567.*
17. L. Rivier and **M. Saugy** (1999) Peptide hormones abuse in sport : State of the art in the detection of growth hormone and erythropoietin. *J. Toxicol. - Toxin reviews 18, 145-176.*
18. **M. Saugy** , Cardis C, Robinson N, Schweizer C (2000) Test methods. Anabolics In *"Baillière's Best Practice and research in clinical endocrinology and metabolism (Special issue on Doping in Sport, P.E. Mullis, Guest Editor) 14, 111-13*3.
19. N Robinson, C Schweizer, C Cardis, **M Saugy**, M Kamber, L Schattenberg, P Mangin (2000), *Heamatological and biochemical parameters from all professionnal cyclists during Tour de Suisse 1999. Schw. Zeitsch. Sport Med und Traum. 48 (3) 104-110*
20. M. Kamber, N. Baume, **M. Saugy** et L. Rivier (2001) Nutritional supplements as a source for positive doping cases ? *Int. J. Sport Nutr. & Exercise Metabolism, 11, 258-269.*
21. K. Fluri, L. Rivier, A. Dienes-Nagy, C. You, A. Maître, C. Schweizer, **M. Saugy**, P. Mangin (2001). Method for confirmation of synthetic corticosteroids in doping urine samples by liquid chromatography - electrospray ionisation mass spectrometry *J. Chromatogr. A.926, 87-95*
22. N Robinson, F Taroni, **M Saugy**, C Ayotte, P Mangin and J Dvorak. (2001). Detection of nandrolone metabolites in urine after a football game in professional and amateur players: a baysian comparison. *Forensic Sci. Int. 122, 130-135.*
23. **M. Saugy**. (2001). Hormone de croissance et sport. *Rev. Fr. Laboratoires, 331, 45-49.*
24. N. Robinson, **M. Saugy**, T. Buclin, G. Gremion, P. Mangin. (2002) The interpretation of secondary blood markers can get hazardous in case of discontinous rhEPO treatment, *Haematologica 87(6). ETL 28.*
25. N. Robinson, **M. Saugy** and P Mangin.(2003) Effects of exercise on the blood markers commonly used to suspect erythropoietin doping. *Clin. Labor. 49 (1-2) 57-62.*
26. L. Avois, P. Mangin and **M. Saugy** (2003) Development and validation of capillary zone electrophoresis method for the determination of ephedrine and relataed compounds in urine without extraction. *J. Chromatography B, 791, 203-216.*
27. N. Robinson, P. Mangin, L. Schattenberg and **M. Saugy** (2003) Origine du suivi hématologique. : les contrôles hématocrite dans le cyclisme. *Rev. Fr. Laboratoires, 350, 53-59.*
28. N. Baume, L. Avois, C. Schweizer, C. Cardis, J. Dvorack, M. Cauderay, P. Mangin and **M. Saugy** (2004) 13C Nandrolone excretion in trained athletes: interindividual variability in metabolism. *Clin. Chem. 50:2, 355-364.*
29. N. Robinson, P. Mangin and **M. Saugy** (2004) Time and temperature dependant changes in red blood cell analytes used for testing recombinant erythropoietin abuse in sport. *Clin. Lab.50, 317-323.*
30. A. Maitre, C. Saudan, P. Mangin and **M. Saugy** (2004). Urinary analysis of four testosterone metabolites and pregnanediol by gas Chromatography/combustion/isotope ratio mass spectrometry after oral administration of testosterone . *J. Anal. Tox 28,6, 426-431.*

31. C. Schweizer, **M. Saugy** and M. Kamber. (2004) Doping test reveals high concentration of salbutamol in a swiss track and field athlete. *Clin J Sport Med. 14, 312-315.*
32. H Zotter, N. Robinson, M. Zorzoli, L. Schattenberg, **M. Saugy** and P. Mangin (2004) Abnormally high serum ferritin level among professional road cyclists. Br J Sports Med 38. 704-708
33. C. Saudan, N. Baume, P. Mangin and **M. Saugy** (2004) Urinary analysis of 16 (alpha)-androsten-3alpha-ol by gas chromatography/combustion/isotope ratio mass spectrometry. Implication in doping analyses. J. Chromatography B 810. 157-164
34. L. Mateus-Avois, P. Mangin, **M. Saugy** (2005) Use of trap gas chromatography-multiple mass spectrometry for the detection and confirmation of 3'-hydroxystanozolol at trace levels in urine for doping control. J. Chromatography B 816 193-201
35. N. Robinson, L. Schattenberg, M. Zorzoli, P. Mangin and **M. Saugy** (2005) Hematological analysis conducted at the departure of Tour de France 2001. Int. J. Sport Med 26 (3) 200-207
36. A. Panchaud, L. Avois, M. Roulet, M. Pilet, C. Hug, **M. Saugy** and L.A. Decosterd (2005) A validated liquid chromatography-mass spectrometry method for the determination of leukotriene B4 and B5 produced by stimulated human polymorphonuclear leukocytes. Anal. Biochem, 341, 58-68
37. C. Saudan, A. Demarchelier, PE Sottas, P. Mangin and **M. Saugy** (2005) Urinary marker of oral pregnenolone administration Steroids 70, 179-183
38. N. Baume, L. Avois, P. Sottas, J. Dvorak, M. Cauderay, P. Mangin and **M. Saugy** (2005) Effect of intensity on 13C-nandrolone excretion in trained athletes Clin. J. Sport Med *15(3):158-66*
39. N. Baume, N. Mahler, M. Kamber, P. Mangin and **M. Saugy** (2005) Research of stimulants and anabolic steroids in dietary supplements Scan J Med Sci Sports *16 (1) ; 41-48*
40. V. Castella, N Dimo-Simonin, C Brandt-Casadevall, N. Robinson, **M. Saugy,** F Taroni and P. Mangin (2005) Forensic identification of urine samples: a comparison between nuclear and mitochondrial DNA markers, Int J Leg Med, *120 (2), 67-72*
41. G. Lippi, M. Franchini, G.C. Guidi, Robinson, N., Mangin, P., **Saugy, M**. (2005) International Journal of Sports Medicine 26 (6), 508-511 (letters to the editor)
42. Saudan, C., Augsburger, M., Kintz, P., **Saugy, M**., Mangin, P (2005) Detection of exogenous GHB in blood by gas chromatography-combustion- isotope ratio mass spectrometry: Implications in postmortem toxicology J Anal Tox *29 (8), 777-781*
43. Baume, N., Mahler, N., Kamber, M., Mangin, P., **Saugy, M.** (2006) Research of stimulants and anabolic steroids in dietary supplements Scand J Med Sci Sports *16 (1), 41-48*
44. Saudan, C., Kamber, M., Barbati, G., Robinson, N., Desmarchelier, A., Mangin, P., **Saugy, M**. (2006) Longitudinal profiling of urinary steroids by gas chromatography/ combustion/isotope ratio mass spectrometry: Diet change may result in carbon isotopic variations J Chrom B *831 (1-2) 324-327*
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5. RENSEIGNEMENTS SUR L'INSTITUTION D'ORIGINE ET LES RESSOURCES DISPONIBLES

Expertise de l'ISSUL, du GRSA et du LAD

Créé en 1995, l'ISSUL (Institut des Sciences du Sport de l'université de Lausanne) est un institut de la Faculté des Sciences Sociales et Politiques de l'Université de Lausanne. Il réunit des experts en sciences sociales et en sciences de la vie.

Les membres de l'équipe de l'ISSUL développent des travaux en sociologie du sport depuis plusieurs années et possèdent une excellente connaissance des recherches internationales. L'équipe possède une bonne maîtrise des méthodes quantitatives et qualitatives. Les expertises portent sur plusieurs domaines. Les travaux portant sur le dopage[1] s'articulent bien avec les perspectives développées sur la consommation et le goût sportif[2] et permettent de mieux comprendre les effets du contexte sur les choix de consommation. L'expertise dans l'analyse des carrières des consommateurs, des effets de l'offre, du rôle des consommations dans les sociabilités, de l'influence de la socialisation secondaire sur la consommation permettra de mieux comprendre les « goûts » et « dégoûts » pour les produits dopants et le passage de l'un à l'autre.

L'ISSUL s'appuiera aussi sur l'équipe d'assistants, en particulier Ronan Coquet qui réalise une thèse sur le dopage en body-building (financement du fonds national suisse de la recherche) et Orlan Moret, qui a travaillé sur la violence, a déjà réalisé des enquêtes de terrain en hockey sur glace et qui connaît bien le milieu puisqu'il joue et à joué au hockey dans des équipes de niveau national. Cette expertise de l'ISSUL est très complémentaire de celles du GRSA (Groupe de Recherche sur la Santé des Adolescents), qui est spécialisée dans la santé des adolescents et possèdes une grande expérience de la recherche sur toutes les formes de conduites addictives, et du LAD (Laboratoire d'Analyse du Dopage), qui est spécialisé dans les approches biologiques et forensiques du dopage (voir les publications de JC Suris et de M. Saugy pour quelques indications sur les compétences).

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6. DOCUMENTS D'EVALUATION ETHIQUE

Processus de recrutement

Nous pouvons bénéficier d'un réseau de joueurs et entraineurs de hockey sur glace pour pénétrer le milieu et faire en sorte que notre travail soit accepté.
Comme dans les travaux précédents nos travaux, nos méthodologies et le but du travail seront précisés.
Lors de cette première phase de mise en confiance, un formulaire de consentement (voir ci-dessous, annexe 1) comprenant les procédures de préservation de l'anonymat est présenté.
Signé en deux exemplaires, il précise les modalités de l'enregistrement audio, de retranscription des données personnelles et d'envoi au sportif concerné. Les modalités d'approche de cette convention d'entretien sont celles utilisées régulièrement à Lausanne. Elles ont permis aux membres de l'équipe de recherche de rencontrer un certain nombre d'anciens sportifs dans différents sports sans qu'il n'y ait jamais eu de problème de préservation de l'anonymat.

Confidentialité et anonymat des réponses
Une fois l'entretien retranscrit les noms, les dates et les lieux sont changés. Dans la convention d'entretien la personne interrogée a la possibilité de relire le texte et d'enlever ce qu'elle désire.

ANNEXE 1 : Formulaire de consentement éclairé

Sous la direction de Fabien OHL, Pr, Institut des Sciences du Sport de l'université de Lausanne (ISSUL), Faculté des Sciences Sociales et Politiques, Université de Lausanne.

La personne soussignée :

1. Certifie avoir été informée des avantages et des risques éventuels qui sont associés à cette étude, et des contraintes qu'impliquait sa participation à cette étude.
2. Atteste qu'un temps de réflexion suffisant lui a été accordé.
3. Certifie avoir été informé sur les objectifs et le déroulement de l'étude ci-dessus, par le chargé d'étude dont le nom figure au bas de cette page.
4. Affirme avoir lu attentivement et compris les informations écrites fournies en annexe, informations à propos desquelles il a pu poser toutes les questions qu'il souhaitait.
5. A été informée du fait qu'elle pouvait interrompre à tout instant sa participation à cette étude sans préjudice d'aucune sorte.
6. A été informée qu'il n'y a aucune obligation de répondre aux questions jugées indiscrètes.
7. A été informée que le magnétophone enregistreur peut être arrêté à tout moment à la demande de la personne interviewée.
8. A été informée qu'entre l'interview et la présentation du rapport, elle peut demander à tout moment de revenir en arrière sur ses autorisations afin que certaines informations soient censurées, que les éléments permettant de le reconnaître soient transformés ou que son entretien ne soit pas publié « publiquement ».
9. A été informée que les enregistrements des interviews seront supprimés une fois l'étude achevée.

COCHER L'OPTION SOUHAITEE ET RAYER LA MENTION INUTILE

Nom de l'interviewer : …………………………..

Nom du répondant : ....

❑ **J'autorise** ❑ **J'interdis** la publication de mon interview.

❑ **Je désire** ❑ **Je ne désire pas** recevoir mon interview retranscrite par écrit et signée (chaque feuille) par l'interviewer.

Cette convention est signée en double exemplaire par l'interviewer et l'interviewé.

Le soussigné accepte de participer à l'étude mentionnée ci-dessus.

Fait à…………………………………., le……………………………………….

Signature du responsable de l'étude, Pr. Ohl : | Signature de l'interviewer : | Signature du répondant :

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