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Journal: Journal of analytical toxicology

Year: 2020 Jan 2

DOI: 10.1093/jat/bkz113

Parallel reaction monitoring-based quantification of cannabinoids in whole blood

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Abstract

Cannabis is the most consumed drug of abuse making it the primary target for identification and quantification in human whole blood regarding forensic and clinical toxicology analyses. Among biological matrices, blood is the reference for toxicological interpretation. A highly sensitive and selective liquid chromatography (LC) hyphenated with high resolution mass spectrometry (HRMS) was developed for the quantification of Δ^9-tetrahydrocannabinol (THC), 11-hydroxytetrahydrocannabinol (THC-OH), 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH), and cannabidiol (CBD). Those cannabinoids were extracted from 1 mL of whole blood by a simple liquid-liquid extraction (LLE) in acidic conditions. HRMS was performed on an Orbitrap-based instrument using its trapping capabilities and increased selectivity for parallel reaction monitoring (PRM) quantification in positive polarity with a negative polarity switching for THC-OH and THC-COOH. Although selected reaction monitoring (SRM) and PRM targeted methods have similar performance in terms of linearity, dynamic range, precision and repeatability, Orbitrap-based PRM provides a higher specificity due to the use of high-resolution mode separating background ions from the targeted molecules. The method was fully validated according to guidelines set forth by the "Société Française des Sciences et des Techniques Pharmaceutiques" (SFSTP). Trueness was measured below 107 % for all tested concentrations. Repeatability and intermediate precision were found to be lower than 12 % while the assay was found to be linear in the concentration range of 0.4-20 ng/ml for THC, THC-OH and CBD and of 2-100 ng/ml for THC-COOH. Recovery (RE) and matrix effect (ME) ranged from 70.6 to 102.5 % and -40 % and 6.6 % respectively. The validated method provides an efficient procedure for the simultaneous and rapid quantification of cannabinoids in PRM mode providing an alternative over classical SRM.

Introduction

Cannabis is a complex plant containing more than 400 compounds including at least 60 cannabinoids (1). Cannabis is the most widespread drug of abuse in the world and its consumption is gaining acceptance both culturally and legally in many countries (2). Despite its psychoactive effects, various uses of cannabis have been described in the clinical world. Indeed, its use has been proposed for the treatment of multiple pathologies, such as multiple sclerosis, epilepsy, neuropathic pain, arthritis, nausea and vomiting, appetite stimulation, anxiety and sleep disorders, psychosis, glaucoma, and Tourette syndrome (3-7). Cannabis plant extracts contains many substances including tetrahydrocannabinolic acid (THC-A) and cannabidiolic acid (CBD-A) non-psychoactive substances that are being decarboxylated into Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) under heat (8). THC is responsible for most of the psychoactive effects and in this context, its accurate quantification is crucial for forensic and clinical purposes to realistically determine its behavioural impact (9).

In humans, THC is mainly metabolized in the liver by cytochrome P450 into two main metabolites: 11-hydroxytetrahydrocannabinol (THC-OH) and 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) (10). The quantification of THC and its two main metabolites in plasma allows the application of mathematical models predicting the time of marijuana consumption (11). In the past years, gas (GC) and high-performance liquid (HPLC) chromatography approaches coupled with mass spectrometry (MS) have been the gold standard regarding cannabinoids analyses (12-14). The heating procedure required for GC analyses leads to unavoidable decarboxylation of the cannabinoid's acids (14). Therefore, a preliminary derivatization step is required to improve the thermal stability and volatility of the compounds of interest (8, 15). On the other hand, LC analyses are performed at limited

temperature preserving the cannabinoids acids in the extracted sample (15). The development of LC-MS systems have allowed the emergence of sensitive and robust methods for the detection of cannabinoids reducing the sample preparation and injection time in a significant manner (16).

Improvements regarding instrumentation and bioinformatics have broadened the number of possible MS-based analytical strategies (17, 18). High-resolution (HR) MS-based parallel reaction monitoring (PRM) has emerged as an alternative method of targeted quantification especially in the field of proteomics. Up to now, Selected Reaction Monitoring (SRM) performed either on triple-quadrupole (QQQ) or hybrid quadrupole-linear ion trap (QTrap) mass spectrometers has been the gold standard regarding targeted quantitative analyses (19, 20). SRM and PRM targeted methods have similar performance in terms of linearity, dynamic range, precision and repeatability (21). Yet, among other advantages, Orbitrap-based PRM provides a higher specificity due to the use of high-resolution mode separating background ions from the targeted molecules (22). An Orbitrap instrument, by nature, is a trapping device where low abundant precursors can be sampled of longer periods of time to measure low abundant precursors and increase the sensitivity (23). PRM implies the acquisition of all selected precursors' fragments facilitating multi fragments approaches. Therefore, very limited *a priori* information (only precursor ion m/z) is required regarding the targeted analytes making this approach more flexible.

PRM applications have been widely described regarding proteomics approaches (17, 22-24), the use of such approaches for the quantitation of small molecules receives a growing interest. Targeted metabolite quantitation in PRM has been described in parallel of untargeted metabolomics (25). Studies in PRM mode have also been reported for the quantitation of drugs such as anticancer (abiraterone) (26) and antiviral drug for the treatment of hepatitis C

(beclabuvir) in plasma (27). Anticoagulant rodenticides (28) and sterols quantitation (29) have also been performed using this analytical strategy.

Herein, we present a fully developed and validated procedure for cannabinoids quantification in whole blood, being the matrix of choice regarding driving under the influence of drugs (DRUID) in Switzerland. The validated procedure is performed on a LC-HRMS system seizing the advantages of PRM that have been assessed for proteomic studies for drugs of abuse.

Materials and methods

Standards and reagents

Water, methanol, formic acid (FA) and ammonium formate were purchased from Biosolve, while n-hexane, ethyl acetate and acetic acid were purchased from Sigma-Aldrich. Drug standards were furnished by Cerilliant or Lipomed, either at 1 mg/mL or 100 µg/mL. Blank lyophilized whole blood was acquired from ACQ Science.

Solution preparation

A standard solution of THC, THC-OH, and CBD was prepared at 100 ng/mL for calibration curve and quality control preparation. In the same purpose, a solution of THC-COOH at 1 µg/mL was made. Finally, a solution containing THC-D3, THC-OH-D3, CBD-D3 at 100 ng/mL and THC-COOH-D9 at 500 ng/mL was prepared as internal standard (IS).

Calibration samples were prepared by spiking lyophilised whole blood at 7 concentration levels ranging from 0.4 to 20 ng/mL regarding THC, THC-OH and CBD and from 2 to 100 ng/mL for THC-COOH.

Sample pre-treatment

One hundred microliters of IS were spiked and evaporated to dryness before adding 1 mL of whole blood. After the addition of 200 µL of acetic acid (10 %), liquid-liquid extraction was performed with 5 mL of hexane/ethyl acetate (9:1 v/v) by slow horizontal shaking for 10 minutes. After centrifugation for 10 min at 4350 rpm, the upper organic phase was transferred into a glass tube and evaporated to dryness under nitrogen. Reconstitution was performed by adding 50 µL of methanol, after vortexing, 50 µL of water was added and 10 µL was injected into the LC-MS system.

LC-HRMS method

Chromatographic separation was performed using a Thermo Scientific Ultimate 3000 LC system with a Phenomenex 2.6 µm C18 (2.1 X 5 mm) maintained at 45°C. Mobile phase A consisted of ammonium formate 5 mM pH 7.7 while mobile phase B consisted of methanol with 0.1 % FA. Phase B was ramped linearly from 50 to 95% over 5 minutes. The column was washed at 98 % of B for 1 minutes, followed by a 2 minutes reequilibration at 2 % of B for a total analysis time of 8 minutes with a 300 µL/min flowrate. The LC was coupled to a Q Exactive Plus system (Thermo Scientific, Bremen, Germany) via a heated electro spray ionisation (ESI) source. The ionisation spray voltage was set to 3 kV, sheath gas flowrate was set to 40 and auxiliary gas flowrate to 10 (both in arbitrary unit). The method operated in PRM using an inclusion list for the specific detection of THC and CBD in positive polarity and THC-OH and THC-COOH in negative polarity with HCD fragmentation using NCE at 50 eV. Polarity was set to positive for the first 4.6 minutes and then switched to negative for the last 3.4 minutes. Resolution was set at 17’500 for the fragmentation experiments with an AGC target of 1e5 and a maximum IT of 100 ms.

Method validation

The performance of the analytical process was evaluated according to the validation criteria based on the "Société Française des Sciences et des Techniques Pharmaceutiques" (SFSTP) directives regarding bioanalytical methods and adapted to our specific requirements (30-32). The validation was carried out over three non-consecutive days (p=3). The method's precision and trueness were established using a statistical treatment based on variance analysis (ANOVA).

During this validation procedure, the analysis of both calibration (Cal) and quality control (QC) whole blood samples was performed. Both the Cal and the QC were prepared in quadruplicate (n=4). Cal were prepared at 7 concentration levels (k=7; Cal=0, 0.4, 1, 2, 5, 10 and 20 ng/mL for THC, THC-OH and CBD and Cal=0, 2, 5, 10, 25, 50, 100 ng/mL for THC-COOH) while QC were prepared independently at 4 concentration levels (k=4; Cal=0.4, 1, 10 and 20 ng/mL for THC, THC-OH and CBD and Cal=2, 5, 50 and 100 ng/mL for THC-COOH). The samples were then analysed according to the procedure detailed earlier. This way, trueness, precision, accuracy, linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined.

Selectivity was assessed analysing six different blank bloods and investigating potential interfering peaks for the reaction monitoring of the substances of interest. Recovery (RE) and matrix effect (ME) were evaluated according to the approach described by Matuszweski et al. (33), 5 replicates of three samples sets were prepared including the 4 substances of interest at two concentration levels (1 and 10 ng/ml for THC, THC-OH and CBD and 5 and 50 ng/ml for THC-COOH). Sample set 1 represented neat standards spiked after extraction. Sample set 2 represented blank matrix spiked after extraction, while sample set 3 represented blank matrix spiked before extraction. Specificity was established as the absence of interfering peaks at the

retention time (RT) of the analyte and IS. Stability was also evaluated at the two concentration levels used for ME and RE evaluation in 5 replicates. Three cycles of freeze-thaw (-20°C), benchtop (6 hour, room temperature), autosampler (24 hours, 5 °C), and short term (1 week, -20°C) conditions were performed in this assay (1, 2). Longer term stability was evaluated by comparing real cases analysis in PRM mode with MRM analysis performed on the same samples between 2 and 4 months before after a conservation at -20 °C.

Finally, 187 DRUID samples from forensic toxicology were analysed both using the developed procedure and an MRM-based published one used in routine using the same extraction procedure (12, 34, 35).

Results and discussion

Method development

Cannabis is the most consumed drug of abuse and cannabinoids-based medications' use is growing reinforcing the need for accurate, sensitive and robust cannabinoids quantification. Cannabinoids quantification is quite challenging mainly due to the high retention on a reversed phase column and the poor extraction yield due to its binding to active surfaces/matrices (36). In this study, THC, THC-OH, THC-COOH and CBD being the primary cannabinoids targeted for forensic analyses were selected to successfully develop an Orbitrap-based PRM quantification method with a sensibility in adequation with the forensic threshold. The process involves a single LLE in acidic conditions as a simple sample preparation (35, 37). The procedure's optimization allows the rapid analysis of those molecules of interest (8 minutes per run) and an increase of the signal to noise ratio (S/N) compared to the MRM analyses (34). The method transfers the widely described PRM advantages for proteomic for small molecules quantification and confirms that PRM is a solid alternative towards classical MRM analysis.

Method validation

In preliminary experiments, simple linear regression model based on the least square method and different weighted linear regression models were evaluated. The goal was to select the most suitable response function by calculating the existing relationship between drug to IS ratio and the expected concentration. The best results were obtained using a regression model with a weight of *1/X*, considering the relationship between the natural logarithm of the response variance and the expected concentration (12). As expected, this regression model showed an interest to increase the statistical weight of the lower concentrations, the simple linear regression model being generally more influenced by the upper concentrations.

Trueness and precision

As previously mentioned, trueness and precision were determined by independent QC samples at 4 different concentration levels injected in quadruplicate over 3 non-consecutive days. The accuracy refers to the total error and comprises two components (38). Trueness indicates the “bias” or systematic errors and can be calculated by comparing the percentage difference between the experimental and the expected theoretical values. The systematic error of the developed method varied from 0.2 to 6 % (Table 1). Precision can be referred to random errors or standard deviation. Two parameters were calculated regarding precision including the relative standard deviation (repeatability) and the between day variability (intermediate precision) (39). Repeatability ($R_{R.S.D.}$) represents the precision under similar conditions when analyses are performed by the same operator using the same reagents and samples. The intermediate precision ($IP_{R.S.D.}$) is calculated using analyses of the same samples under different conditions (different days and reagents) For both those parameters, values were comprised between 1.5 and 11.2 % (Table 1).

Accuracy considers the total error including both the systematic and the random errors. Accuracy profiles illustrate the uncertainty measurement represented using trueness, confidence limits at 95 % of the total measurement error (precision) calculated for each concentration level and the acceptance limits of ± 20 % suggested for method validation at the LLOQ (±15 % at other concentration levels) (30). All analysed QCs were within the acceptance limits (Figure 1).

Linearity and LOQ

Linearity is defined as the method capacity to deliver a result proportional to the concentration within the sample (12). A linear regression model based on the least square method was applied on the fit of the back-calculated concentration as a function of the expected concentration. Values for the coefficient of determination were above 0.9964 for the four substances of interest (Table 1). Slope values were comprised between 0.9917 and 1.013 confirming the method linearity within the concentration ranges of interest. For the method forensic purpose, LOQ was fixed at 0.4 ng/ml for THC, CBD and THC-OH and 2.5 ng/ml for THC-COOH. At those concentrations the accuracy profiles were comprised within the ± 20 % acceptance limit at LLOQ.

Selectivity, recovery and matrix effect

Selectivity is defined as the method ability to measure unambiguously and to differentiate the analyte of interest towards potential interferences. No effects were observed extracting and analysing 6 different blank blood samples meaning that no interfering compounds did impair the detection nor the quantification of the substances of interest. One of the reasons might be the use of HRMS since by improving the mass resolution power, this technology increases the selectivity, therefore reducing the number of potential interferences (40). Nevertheless, selectivity is often challenged due to ion suppression or enhancement caused by the matrix

especially using ESI ion sources (33). Therefore, the assessment of such ME is crucial especially regarding quantitative approaches. ME was determined to be between 40% (10% CV) of ion suppression for THC in high concentration and 6.6 % of ion enhancement (16,9 % CV) for THC-OH. The values for both ME and RE are summarized in Table 2. Those results characterized by an ion suppression for THC and CBD are in adequation with the existing literature (41-43). Those relatively high values of ME can be due to residual matrix components in the final extract affecting the ionization process. The addition of an isotopically labelled IS with similar physico-chemical properties is used herein to efficiently compensate those ME.

Stability

The results of stability evaluation are presented in Table 3. Stability of the method was assessed at two different concentrations (1 and 10 ng/ml for THC, CBD and THC-OH and 5 and 50 ng/ml for THC-COOH). The mean stability in terms of accuracy ranged from 96.7 % to 104 %, 93.5 % to 102.1%, 92.2% to 99.4 % and 92% to 99.2 % for auto-sampler (24 hours, 5°C), three cycles of freeze-thaw (-20°C), bench-top (6 hours, room temperature), and short-term stability (1 week, -20°C) respectively.

Real samples analysis

Among the 187 analysed samples, THC was above the used limits of decision (1 ng/ml for THC, CBD and THC-OH and 5 ng/ml for THC-COOH) in 139 cases while this number of positive cases decreases to 136, 99 and 25 regarding THC-COOH, THC-OH and CBD respectively. The concentration distribution of all four molecules of interest after PRM analysis is represented in Figure 2. Results comparison between the described method and a previously published MRM method (35) are represented in Figure 3 for THC, THC-OH and THC-COOH. CBD was measured above the used limits of detection in only 25 cases,

therefore we did not represent the correlation regarding CBD in Figure 3. A good correlation was observed between the PRM and the MRM values. The relative standard deviation was lower than 20 % for all four substances in almost every compared sample confirming the suitability of PRM quantitative approaches for small molecules analysis. The calculated Spearman correlation coefficient measuring the linear correlation between the two conditions were above 0.993 for all four measured cannabinoids.

Conclusion

A rapid, sensitive, reproducible and robust method was developed for the detection and quantification of THC, THC-OH, THC-COOH and CBD. This study present PRM as an interesting alternative to MRM classical quantitative approaches constituting a true force for toxicological analysis with forensic purpose. It confirms that MRM and PRM targeted methods provide comparable sensitivity, linearity, dynamic range, precision and repeatability (22). PRM analyses give access to full MS/MS spectra not focusing on a specific transition allowing to work without *a priori* knowledge of the fragments of interest and leading to an increased control over the quantification experiment. Exploiting Orbitrap high resolving power and their trapping capabilities offer a clear advantage being an alternative over triple quadrupole instruments.

Acknowledgments

The authors acknowledge financial support from the Swiss Federal Office of Public Health (contract no. 14.013792.204.0001/1300).

References

1. Ravula, A., Chandasana, H., Setlow, B., Febo, M., Bruijnzeel, A.W., Derendorf, H. (2018) Simultaneous quantification of cannabinoids tetrahydrocannabinol, cannabidiol and CB1 receptor antagonist in rat plasma: An application to characterize pharmacokinetics after passive cannabis

smoke inhalation and co-administration of rimonabant. *Journal of Pharmaceutical and Biomedical Analysis*, **160**, 119-125.
2. Jamwal, R., Topletz, A.R., Ramratnam, B., Akhlaghi, F. (2017) Ultra-high performance liquid chromatography tandem mass-spectrometry for simple and simultaneous quantification of cannabinoids. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, **1048**, 10-18.
3. Koppel, B.S., Brust, J.C., Fife, T., Bronstein, J., Youssof, S., Gronseth, G.*, et al.* (2014) Systematic review: efficacy and safety of medical marijuana in selected neurologic disorders: report of the Guideline Development Subcommittee of the American Academy of Neurology. *Neurology*, **82**, 1556-1563.
4. Borgelt, L.M., Franson, K.L., Nussbaum, A.M., Wang, G.S. (2013) The pharmacologic and clinical effects of medical cannabis. *Pharmacotherapy*, **33**, 195-209.
5. Blake, D.R., Robson, P., Ho, M., Jubb, R.W., McCabe, C.S. (2006) Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis. *Rheumatology*, **45**, 50-52.
6. Whiting, P.F., Wolff, R.F., Deshpande, S., Di Nisio, M., Duffy, S., Hernandez, A.V.*, et al.* (2015) Cannabinoids for Medical Use: A Systematic Review and Meta-analysis. *Journal of the American Medical Association*, **313**, 2456-2473.
7. Baron, E.P. (2015) Comprehensive Review of Medicinal Marijuana, Cannabinoids, and Therapeutic Implications in Medicine and Headache: What a Long Strange Trip It's Been. *Headache*, **55**, 885-916.
8. Burnier, C., Esseiva, P., Roussel, C. (2019) Quantification of THC in Cannabis plants by fast-HPLC-DAD: A promising method for routine analyses. *Talanta*, **192**, 135-141.
9. Lupica, C.R., Riegel, A.C., Hoffman, A.F. (2004) Marijuana and cannabinoid regulation of brain reward circuits. *British Journal of Pharmacology*, **143**, 227-234.
10. Watanabe, K., Yamaori, S., Funahashi, T., Kimura, T., Yamamoto, I. (2007) Cytochrome P450 enzymes involved in the metabolism of tetrahydrocannabinols and cannabinol by human hepatic microsomes. *Life Sciences*, **80**, 1415-1419.
11. Huestis, M.A., Barnes, A., Smith, M.L. (2005) Estimating the time of last cannabis use from plasma delta9-tetrahydrocannabinol and 11-nor-9-carboxy-delta9-tetrahydrocannabinol concentrations. *Clinical Chemistry*, **51**, 2289-2295.
12. Thomas, A., Widmer, C., Hopfgartner, G., Staub, C. (2007) Fast gas chromatography and negative-ion chemical ionization tandem mass spectrometry for forensic analysis of cannabinoids in whole blood. *Journal of Pharmaceutical and Biomedical Analysis*, **45**, 495-503.
13. Leghissa, A., Hildenbrand, Z.L., Schug, K.A. (2018) A review of methods for the chemical characterization of cannabis natural products. *Journal of Separation Science*, **41**, 398-415.
14. Citti, C., Braghiroli, D., Vandelli, M.A., Cannazza, G. (2018) Pharmaceutical and biomedical analysis of cannabinoids: A critical review. *Journal of Pharmaceutical and Biomedical Analysis*, **147**, 565-579.
15. Hazekamp, A., Peltenburg, A., Verpoorte, R., Giroud, C. (2005) Chromatographic and spectroscopic data of cannabinoids from Cannabis sativa L. *Journal of Liquid Chromatography & Related Technologies*, **28**, 2361-2382.
16. Teixeira, H., Verstraete, A., Proenca, P., Corte-Real, F., Monsanto, P., Vieira, D.N. (2007) Validated method for the simultaneous determination of Delta9-THC and Delta9-THC-COOH in oral fluid, urine and whole blood using solid-phase extraction and liquid chromatography-mass spectrometry with electrospray ionization. *Forensic Science International*, **170**, 148-155.
17. Hoffman, M.A., Fang, B., Haura, E.B., Rix, U., Koomen, J.M. (2018) Comparison of Quantitative Mass Spectrometry Platforms for Monitoring Kinase ATP Probe Uptake in Lung Cancer. *Journal of Proteome Research*, **17**, 63-75.

18. Joye, T., Sidibe, J., Deglon, J., Karmime, A., Sporkert, F., Widmer, C.*, et al.* (2019) Liquid chromatography-high resolution mass spectrometry for broad-spectrum drug screening of dried blood spot as microsampling procedure. *Analytica Chimica Acta*, **1063**, 110-116.
19. Hopfgartner, G., Varesio, E., Tschappat, V., Grivet, C., Bourgogne, E., Leuthold, L.A. (2004) Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules. *Journal of Mass Spectrometry*, **39**, 845-855.
20. Stahl-Zeng, J., Lange, V., Ossola, R., Eckhardt, K., Krek, W., Aebersold, R.*, et al.* (2007) High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. *Molecular & Cellular Proteomics*, **6**, 1809-1817.
21. Ronsein, G.E., Pamir, N., von Haller, P.D., Kim, D.S., Oda, M.N., Jarvik, G.P.*, et al.* (2015) Parallel reaction monitoring (PRM) and selected reaction monitoring (SRM) exhibit comparable linearity, dynamic range and precision for targeted quantitative HDL proteomics. *Journal of Proteomics*, **113**, 388-399.
22. Rauniyar, N. (2015) Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry. *International Journal of Molecular Sciences*, **16**, 28566-28581.
23. Domon, B., Gallien, S. (2015) Recent advances in targeted proteomics for clinical applications. *Proteomics Clinical Applications*, **9**, 423-431.
24. Bourmaud, A., Gallien, S., Domon, B. (2016) Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: Principle and applications. *PROTEOMICS*, **16**, 2146-2159.
25. Gao, Y., Chen, Y., Yue, X., He, J., Zhang, R., Xu, J.*, et al.* (2018) Development of simultaneous targeted metabolite quantification and untargeted metabolomics strategy using dual-column liquid chromatography coupled with tandem mass spectrometry. *Analytica Chimica Acta*, **1037**, 369-379.
26. Bhatnagar, A., McKay, M.J., Crumbaker, M., Ahire, K., Karuso, P., Gurney, H.*, et al.* (2018) Quantitation of the anticancer drug abiraterone and its metabolite Delta(4)-abiraterone in human plasma using high-resolution mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, **154**, 66-74.
27. Jiang, H., Titsch, C., Zeng, J., Jones, B., Joyce, P., Gandhi, Y.*, et al.* (2017) Overcoming interference with the detection of a stable isotopically labeled microtracer in the evaluation of beclabuvir absolute bioavailability using a concomitant microtracer approach. *Journal of Pharmaceutical and Biomedical Analysis*, **143**, 9-16.
28. Gao, X., Li, H., Li, H., Dong, S., Chu, J., Guo, H.*, et al.* (2018) Sensitive determination of nine anticoagulant rodenticides in blood by high resolution mass spectrometry with supported liquid extraction pretreatment. *Forensic Science International*, **292**, 39-44.
29. Schott, H.F., Krautbauer, S., Horing, M., Liebisch, G., Matysik, S. (2018) A Validated, Fast Method for Quantification of Sterols and Gut Microbiome Derived 5alpha/beta-Stanols in Human Feces by Isotope Dilution LC-High-Resolution MS. *Analytical Chemistry*, **90**, 8487-8494.
30. Lynch, K.L. (2016) CLSI C62-A: A New Standard for Clinical Mass Spectrometry. *Clinical Chemistry*, **62**, 24-29.
31. Peters, F.T., Drummer, O.H., Musshoff, F. (2007) Validation of new methods. *Forensic Science International*, **165**, 216-224.
32. Boulanger, B., Chiap, P., Dewe, W., Crommen, J., Hubert, P. (2003) An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progress and limitations. *Journal of Pharmaceutical and Biomedical Analysis*, **32**, 753-765.
33. Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M. (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, **75**, 3019-3030.
34. Fabritius, M., Augsburger, M., Chtioui, H., Favrat, B., Giroud, C. (2014) Fitness to drive and cannabis: validation of two blood THCCOOH thresholds to distinguish occasional users from heavy smokers. *Forensic Science International*, **242**, 1-8.

35. Fabritius, M., Chtioui, H., Battistella, G., Annoni, J.M., Dao, K., Favrat, B.*, et al.* (2013) Comparison of cannabinoid concentrations in oral fluid and whole blood between occasional and regular cannabis smokers prior to and after smoking a cannabis joint. *Analytical and Bioanalytical Chemistry*, **405**, 9791-9803.
36. Thomas, A., Geyer, H., Schanzer, W., Crone, C., Kellmann, M., Moehring, T.*, et al.* (2012) Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Analytical and Bioanalytical Chemistry*, **403**, 1279-1289.
37. Nadulski, T., Sporkert, F., Schnelle, M., Stadelmann, A.M., Roser, P., Schefter, T.*, et al.* (2005) Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract. *Journal of Analytical Toxicology*, **29**, 782-789.
38. Gonzalez, A.G., Herrador, M.A., Asuero, A.G. (2010) Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards. *Talanta*, **82**, 1995-1998.
39. Rozet, E., Hubert, C., Ceccato, A., Dewe, W., Ziemons, E., Moonen, F.*, et al.* (2007) Using tolerance intervals in pre-study validation of analytical methods to predict in-study results. The fit-for-future-purpose concept. *Journal of Chromatography A*, **1158**, 126-137.
40. Chindarkar, N.S., Wakefield, M.R., Stone, J.A., Fitzgerald, R.L. (2014) Liquid chromatography high-resolution TOF analysis: investigation of MSE for broad-spectrum drug screening. *Clinical Chemistry*, **60**, 1115-1125.
41. Tiscione, N.B., Miller, R., Shan, X., Sprague, J., Yeatman, D.T. (2016) An Efficient, Robust Method for the Determination of Cannabinoids in Whole Blood by LC-MS-MS. *Journal of Analytical Toxicology*, **40**, 639-648.
42. Palazzoli, F., Citti, C., Licata, M., Vilella, A., Manca, L., Zoli, M.*, et al.* (2018) Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) method for the determination of cannabidiol (CBD), Delta(9)-tetrahydrocannabinol (THC) and its metabolites in rat whole blood after oral administration of a single high dose of CBD. *Journal of Pharmaceutical and Biomedical Analysis*, **150**, 25-32.
43. Schwope, D.M., Scheidweiler, K.B., Huestis, M.A. (2011) Direct quantification of cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, **401**, 1273-1283.

Figure 1: Accuracy profiles for THC, CBD, THC-OH and THC-COOH with a weigthed linear regression model of 1/X. Trueness is represented by a continuous line while the upper and lower accuracy limits and the tolerance limits are represented by dashed lines and dotted lines respectively.

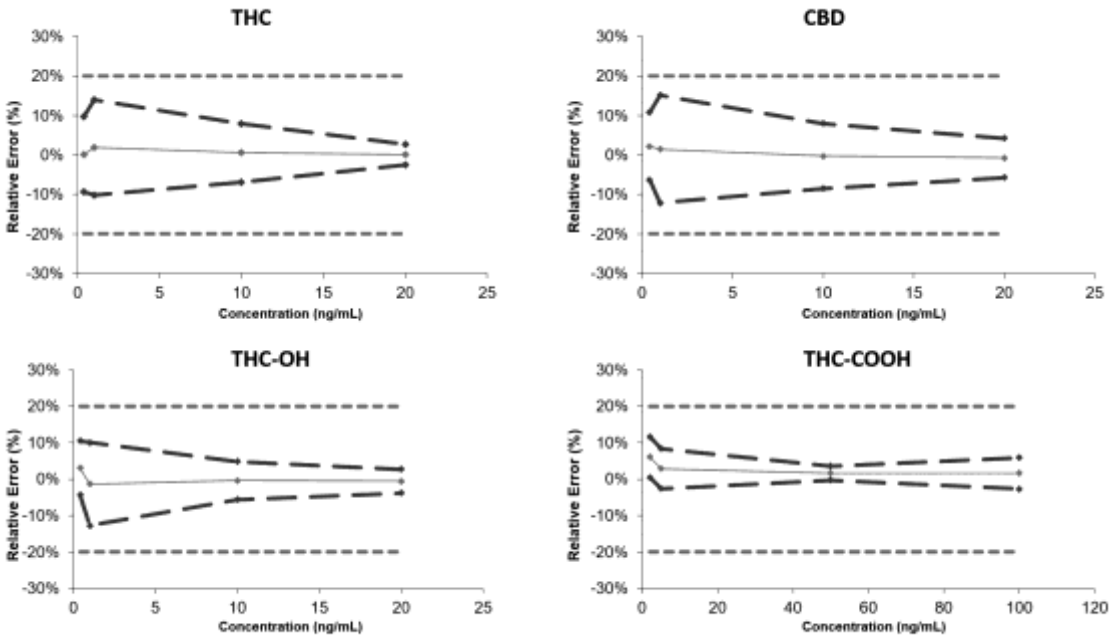

Figure 2: Concentration distribution of the 139 real samples PRM analysis for the four cannabinoids of interest.

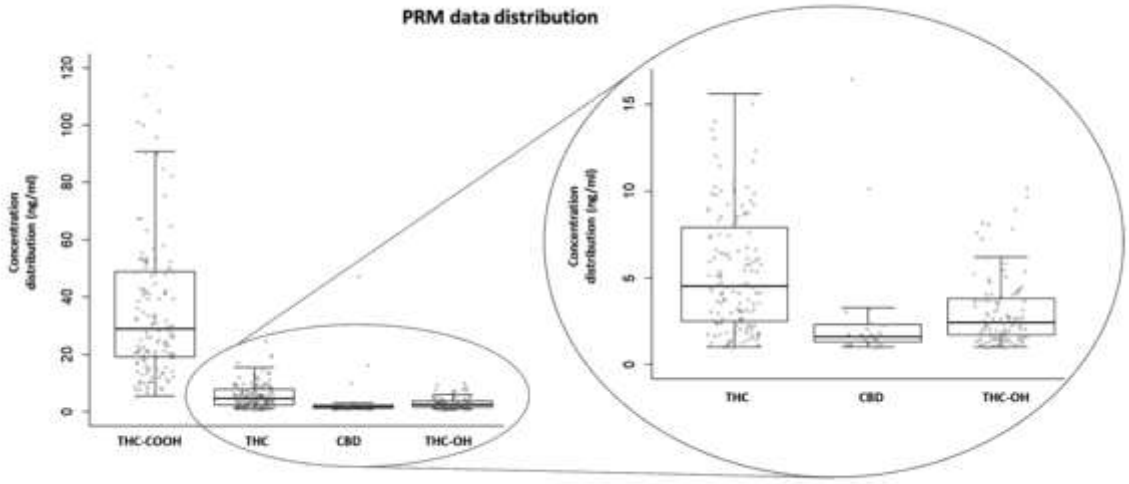

Figure 3: Method comparison for THC, THC-OH and THC-COOH. PRM measured concentrations are plotted as a function of MRM measured concentration using a similar linear regression model. The red lines represent the tolerance limits.

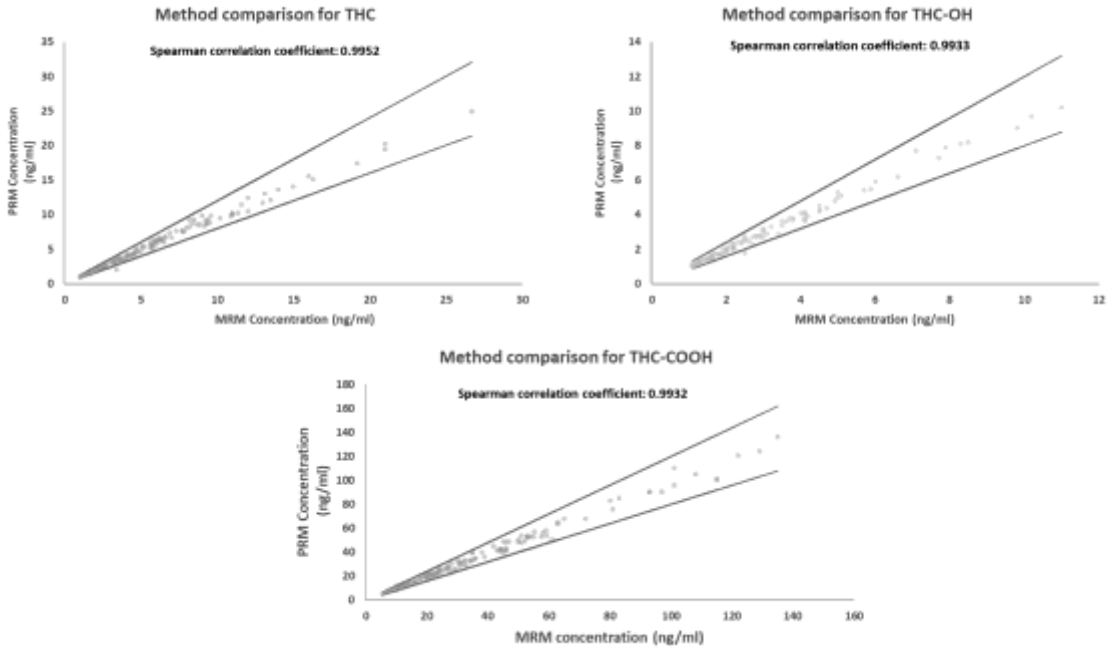

Table 1: Validation results for trueness, precision and linearity (k is the number of concentration levels, n the number of repetitions by levels, and p the number of non-consecutive days)

Trueness (%) **(k=4; n=4; p=3)**				
Calibration level (ng/ml)	**THC**	**CBD**	**THC-OH**	**THC-COOH**
0.4	100.2	102.2	103.1	-
1	101.9	101.5	98.7	-
2	-	-	-	106
5	-	-	-	102.9
10	100.6	99.7	99.6	-
20	100.2	99.2	99.5	-
50	-	-	-	101.7
100	-	-	-	101.6
Precision **Repeatability/Intermediate precision (RSD %)**				
0.4	8.3/8.9	8.1/8.1	7.0/7.0	-
1	3.3/9.9	3.9/11.2	6.9/9.7	-
2	-	-	-	2.6/4.9
5	-	-	-	3.8/4.7
10	2.9/6.5	2.8/7.2	4.1/4.8	-
20	1.6/2.2	2.5/4.3	3.0/3.0	-
50	-	-	-	1.6/1.8

100	-	-	-	1.5/3.6
Linearity				
Range (ng/ml)	0.4-20	0.4-20	0.4-20	2-100
Slope	1.0014	0.9917	0.9945	1.013
Intercept	0.0155	0.0252	0.0058	0.1066
R^2	0.9981	0.9964	0.9979	0.9987
LOQ (ng/ml)	0.4	0.4	0.4	2

Table 2: Results for recovery and matrix effect performed at low- and high-quality control concentrations

	Low Concentration				High Concentration			
Matrix Effect and recovery	**THC (1 ng/ml)**	**CBD (1 ng/ml)**	**THC-OH (1 ng/ml)**	**THC-COOH (5 ng/ml)**	**THC (10 ng/ml)**	**CBD (10 ng/ml)**	**THC-OH (10 ng/ml)**	**THC-COOH (50 ng/ml)**
ME (%)	-34.0	-22.4	6.4	5.2	-40	-21.0	6.60	4.70
CV (%)	18.7	11.3	10.5	12.3	10.0	8.3	16.9	12.3
RE (%)	70.6	75.3	90.7	100.2	87.2	85.0	93.6	102.5
CV (%)	7.0	7.0	13.2	9.3	15.8	13.0	5.4	9.6

Table 3: Three cycles of freeze-thaw (-20°C), benchtop (6 hour, room temperature), autosampler (24 hours, 5 °C), and short term (1 week, -20°C) conditions were performed in this stability assay at low and high quality control concentrations

	Low concentration				**High concentration**			
Stability	**THC (1 ng/ml)**	**CBD (1 ng/ml)**	**THC-OH (1 ng/ml)**	**THC-COOH (5 ng/ml)**	**THC (10 ng/ml)**	**CBD (10 ng/ml)**	**THC-OH (10 ng/ml)**	**THC-COOH (50 ng/ml)**
Auto-sampler (%)	99.0	99.4	101.4	98.8	99.0	98.5	104.0	96.7
Freeze-thaw (%)	99.1	100.4	97.7	97.7	102.1	93.5	98.0	95.4
Bench-top (%)	94.1	97.5	97.3	95.5	92.2	99.4	97.5	92.8
Short term (%)	97.0	92.4	98.8	92.0	99.2	95.6	92.1	95.1