UNIVERSITE DE LAUSANNE – FACULTE DE BIOLOGIE ET DE MEDECINE

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DES PROBLEMES PRE-ANALYTIQUES A L'OXYDATION DES PROTEINES : VOYAGE PROTEOMIQUE

THESE

préparée sous la direction du Professeur ad personam Jean-Daniel Tissot
avec la collaboration de Niels Lion (chargé de cours de l'EPFL)

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RAPPORT DE SYNTHESE

Cette thèse a pour but de démontrer que les protéines sanguines sont sensibles à leur micro-environnement *redox* et que les outils protéomiques permettent pour une part de caractériser les effets de ce micro-environnement au niveau moléculaire. Elle est divisée en trois parties, les deux premières sous forme d'articles de revue publiés et la troisième sous forme d'un travail de recherche mené au Centre de Transfusion Sanguine de Lausanne en collaboration avec la PAF (Protein Analysis Facility) de l'UNIL.

L'article « Plasma/serum proteomics : preanalytical issues » publié dans *Expert Review of Proteomics* en 2007 explique comment la protéine porte l'empreinte de la phase pré-analytique. La technique d'électrophorèse bi-dimensionnelle « différentielle » permet de simplifier cette phase en soumettant tous les échantillons analysés aux mêmes manipulations, ramenant ainsi les variables pré-analytiques aux plus élémentaires d'entre elles.

Dans l'article « Oxidation of proteins : basic principles and perspectives for blood proteomics » publié dans *Proteomics Clinical Applications* en 2008, il est question de l'oxydation comme réaction chimique à l'origine de lésions protéiques. Celles-ci peuvent donner lieu à des artefacts d'analyse protéomique et rendre l'identification de peptides confondante. Elles peuvent par ailleurs être chimiquement instables et s'associer à d'autres composés contenus dans l'échantillon.

Le travail de recherche décrit l'étude protéomique des modifications oxydatives au niveau du fibrinogène oxydé *in vitro*. Les résultats de cette étude indiquent que les conditions de conservation de plasma destiné à la transfusion peuvent potentiellement altérer la structure et la fonction des protéines contenues dans ce produit sanguin et que ce phénomène est pour une part oxydatif.

Plasma/serum proteomics: pre-analytical issues

Stefano Barelli, David Crettaz, Lynne Thadikkaran, Olivier Rubin and Jean-Daniel Tissot†

High-throughput proteomics technologies tend to provide highly sensitive information about living tissues and biological fluids. Analytes are characterized by intrinsic and extrinsic properties, the latter depending on each phase of their preparation, sometimes adding artifacts with crucial repercussions in result reliability and interpretation. This review aims to address some issues that can be encountered when handling plasma and serum in experimental and clinical proteomic settings.

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The enthusiasm for biomarkers research is illustrative of the successes and potential stemming from omics sciences. While genomics refers to DNA whose sequences are considered to be stable databases, proteomics tends to focus on its biological expression. A combination of both provides a holistic approach that is necessary for understanding the intimate mechanisms underlying either physiological or pathological states [1–5].

Blood constitutes an infinite source of analytes that may be potential biomarkers. It is readily accessible and reflects secondary systemic changes that have been evaluated by various techniques over the years. The separation of polypeptides by electrophoresis enabled Arne Tiselius to describe protein fractions corresponding to albumin and α-, β- and γ-globulins in serum. The first diagram of human serum protein electrophoresis was published in 1939. The number of fractions slowly expanded into electrophoretic subfractions, identified as α_1, α_2, β_1, β_2, γ_1 and γ_2. These fractions, characterized by their mobility, are still used to denote serum proteins, such as α_1-macroglobulin, α_2-antiplasmin or β_2-microglobulin. Sophisticated new electrophoretic techniques for identifying many proteins simultaneously and relating them to diseases have been developed thus far and have found applications for almost all body fluids. Despite the developments and progress achieved in protein separation sciences, only a restricted number of methods are routinely used in clinical laboratories. At present, serum protein electrophoresis is mainly used to study major serum protein alterations, such as those observed in patients with inflammatory, liver or kidney diseases, as well as in patients presenting with lymphoproliferative disorders and alterations of immunoglobulin production [6].

With the achievements of proteomic technologies, the search of biomarkers rapidly and widely developed over the last few years. The biological evolution implies important consequences for both the individual and the society [7,8]. The following important questions are arising [9]:

- What is the reliability of such biomarkers?
- How can proteomic profiles express pathological processes?
- Are they related and specific to a particular disease?
- What is the impact of bias on results?

The controversy about ovarian cancer screening provides an illustrative example of skepticism toward promise [10–12]. Elements of response can be found in the methodological parts defining the steps from the sample collection to the analysis, namely, pre-analytics.

Last year, the US FDA, in association with other institutions, announced the Oncology Biomarker Qualification Initiative, with the aim to improve the validation process of particular biomarkers [13]. Clearly, there is a

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need for better standards in clinical proteomics [14]. Many aspects related to pre-analytical issues must be considered when studying proteins in serum and plasma [15,16]. In this review, we will focus on some of these issues, and try to illustrate the mechanism by which they may interfere with the analytical process.

Either in an experimental or clinical setting, the question dictates the choice of the test. The samples to be analyzed have to be manipulated and treated in such a way that will enhance the sensitivity and specificity of the test, keeping in mind that the sample carries the memory of its handling. Samples are influenced by many factors, such as blood sampling, type of tube used for the collection, centrifugation and transportation. Other variables, such as intersubject differences or collection protocol adherence, also potentially alter the integrity of the samples. Many of these variables have been well documented in established references texts [17,18].

Plasma/serum preparation

Plasma is obtained by centrifugation of blood. Centrifugation could represent a source of mechanical stress for blood cells, resulting not only in cell activation, but also in the loss of the plasma membrane integrity. Miyazaki and colleagues studied platelet behavior under high shear-stress conditions [19]. Flow cytometry analyses of the binding of annexin V antibodies to platelets revealed that high shear stress could increase platelet procoagulant activity. This was due to the translocation of phosphatidylserine from the inner leaflet to the outer surface of membrane, leading to the shedding of microparticles. Production of these microparticles was even more rapid by high shear stress than by the combination of two strong platelet agonists, thrombin and collagen. These results suggest that various centrifugation protocols may affect the composition of plasma owing to cell damage.

The use of an anticoagulant is a prerequisite for plasma collection, and is added to the blood collection devices prior to blood withdrawal. After binding Ca^{2+}, EDTA and citrate inhibit the coagulation cascade. Heparin binds to and modifies the activity of the serine protease inhibitor antithrombin (antithrombin III), which inhibits the activated forms of Factor X (Xa) and of Factor II (thrombin). Plasma samples should be rapidly processed after centrifugation. Proteomics studies of plasma and serum revealed several differences, notably in the range of the low-molecular-weight proteome. Multidimensional analyses revealed that EDTA and citrate plasma peptide displays were similar, but were different in the presence of heparin [20]. Using other proteomics approaches, Hsieh and colleagues demonstrated that the use of EDTA resulted in a divergent plasma protein profile when compared with samples anticoagulated with citrate or heparin [21]. In addition, sets of peptides appeared to originate from platelets, adding to the complexity of the pre-analytics. This observation possibly reflects adhesion and aggregation of platelets in the presence of EDTA [22]. In addition to these variables, Baumann and colleagues studied the influence of repeated freeze/thaw cycles on signal intensities in pooled serum and reported a significant impact on the low-molecular-weight peptidome [23].

Clot formation represents a kind of protease storm perturbing proteome profile, evidenced by the presence of additional mass peaks. Furthermore, new interactions between thrombin generation and complement activation pathways have been identified, adding to the intricacy of the protease cascades involved in either coagulation, fibrinolysis or complement activation [24]. According to various studies, approximately 40% of peptides in serum were serum specific [14,20], and most likely resulted from serine proteases involved during coagulation. In addition, it was demonstrated that the composition of serum and plasma related to blood cells changes over time [25,26]. These changes were attributed to plasma membrane pump failure with intracellular glucose depletion, osmotic changes with water movements into the blood cells, as well as to leakage of intracellular peptides.

Significant changes were observed in the proteome of samples undergoing varied clotting times or time lags before centrifugation [21]. This leads to the release of peptides and degraded proteins from blood cells [27]. To avoid such processes, a rapid separation and an immediate freezing of the sample is mandatory, but is frequently associated with logistic problems. An alternative is the addition of protease inhibitors, but their use remains controversial [28]. Surrogate markers demonstrating pre-analytic troubles remain to be identified. Preliminary evidence for the existence of such a marker was provided by Findeisen and colleagues, who observed a time-dependant decrease of a 1467-Da peak during storage of serum samples [29]. The peptide was identified as the N-terminal-truncated fibrinopeptide A.

Selected examples in which pre-analytical steps are crucial

Blood cells release different kinds of cytokines, depending on the type of anticoagulant. Cytokines are usually undetected because of their short half-life and their very low concentrations [30]. An *in vitro* model of cytokine production and protein secretion was proposed by Engstad and colleagues to test the effect of commonly used anticoagulants on monocytes, polymorphonuclear cells and platelets in human whole blood [31]. Ions such as Ca^{2+} were shown to be essential for signal-transduction processes. Their depletion explained why EDTA and citrate inhibited activation of monocytes and polymorphonuclear cells, and suppressed platelet degranulation in whole blood. When cytokine levels were measured in whole blood by immunoassays, they were found to be more stable if the samples were anticoagulated with EDTA, rapidly separated and stored at 4°C [32]. At room temperature, the secretion of cytokines by mononuclear leukocytes appeared to be significantly decreased with citrate phosphate dextrose anticoagulation [33].

The assessment of Down's syndrome during pregnancy usually includes the measurements of α-fetoprotein, unconjugated estriol, total human chorionic gonadotrophin and inhibin-A in serum. However, results can be interpreted with confidence

only if the serum samples were separated within 1 h of draw, kept refrigerated until shipment and tested within 1 week [34]. The stability of carbohydrate-deficient transferrin (used to detect alcohol abuse) was evaluated according to various storage conditions [35]. In samples stored at room temperature, asialo-transferrin and disialo-transferrin decreased by approximately 50%. The correct diagnosis of hemophilia A and von Willebrand's disease also depends on several prenanalytic variables. A so-called 'cold activation' phenomenon, due to the cold storage of whole blood samples before centrifugation, appeared to lead to a significant loss of coagulation Factor VIII and von Willebrand factor [36]. Based on the fact that chilling platelets rearranges the surface configuration of glycoprotein Ib, which is a platelet adhesion receptor, Böhm and colleagues hypothesized that temperature-dependent interactions between glycoprotein Ib and von Willebrand factor was a possible mechanism for the cold-induced loss in von Willbrand factor and Factor VIII [37,38]. The use of fresh-frozen plasma in transfusion medicine also raises pre-analytical questions, leading to questioning the methods by which such products are prepared [39,40]. In this situation, several parameters, such as the length of blood collection, the type of anticoagulant, the centrifugation conditions and the temperature of the process or the removal of residual leukocytes by filtration, are all of major importance.

Pre-analytical issues in mass spectrometry studies

The lack of knowledge of the mechanisms by which a result is generated impedes their verification by an independent technology [41]. The accuracy of data obtained using mass spectrometry (MS) tools is highly dependent on pre-analytical procedures. The design of the studies is important in order to optimize the noise-to-signal ratio and to get reliable results. Some aspects related to MS studies have already been evaluated in detail. For instance, commercially available test tubes may contain silicones, surfactants, polymeric gels or cocktails of protease inhibitors. Thus, it is important to identify these molecules in order to eliminate confounding peaks in peptide mass fingerprint analysis. The MS study of saline solutions incubated in different tubes revealed the appearance of complex series of peaks in the 1000–3000 mass-to-charge ratio (m/z) range, except when collected in glass tubes without additive [42]. The use of polymeric polypropylene tubes was not associated with the generation of interfering signals [23]. When tracking variables intervening in sample handling, Villanueva and colleagues recommended the use of automated platforms [43,44]. Pre-analytical conditions, from the type of collection tube to the spectrometer, must be precisely defined. A major advantage of automation clearly stands in its reproducibility and in the ease of controlling the main pre-analytical and analytical steps [45].

High-abundance proteins

Plasma is known to be a 'haystack' of proteins distributed on a dynamic concentration range of at least 9 orders of magnitude [46,47]. High-abundance components include proteins, such as albumin, immunoglobulins, fibrinogen, transferrin, α_2-macroglobulin, α_1-antitrypsin, haptoglobin and C3 complement. They represent 90% of whole proteins in plasma. Their removal dramatically improves proteome profiling by reducing the complexity of the sample and allows an easier detection of subtle differences between samples. Many approaches have been proposed over the years and have been reviewed [48]. Electrophoretic pre-fractionation techniques permit investigators to concentrate hidden proteins and enables their detection [49]. However, the most commonly used approach is the use of multiple affinity columns [50–58]. These techniques have proved to be useful in many different proteomic studies of plasma. However, one of the most important limitations is the risk of eliminating low-abundance proteins. To reduce this risk, Michel and colleagues proposed different strategies by using multiple affinity columns and electrophoretic fractionation, individually or in combination, prior to MS analysis. In a very interesting study, Gundry and colleagues investigated what they called the 'albuminome' and demonstrated that this protein is able to bind many different peptides [59]. Thus, all separation techniques with the aim to remove albumin (size-exclusion chromatography, antihuman serum albumin antibody affinity chromatography and ion-exchange) or other abundant proteins should be evaluated in depth before being used for biomarker discovery [60].

Centrifugal ultrafiltration in the presence of solvent buffers appeared to be a convenient avenue for removing high-molecular-weight species, without losing the low-molecular-weight species [61]. To deplete high-molecular-weight serum proteins, Tanaka and colleagues evaluated a fully automated device based on multistage filtration [62]. After 1 h of operation, they obtained a sharp protein separation, enabling the detection of many low-abundance peptides. When filtration was performed at low temperature in the presence of protease inhibitors, the mass distribution pattern did not change, suggesting that proteolytic fragmentation was not a main problem. Thulasiraman and colleagues presented an elegant depletion methodology consisting of the saturation–overloading principle [63]. This principle is based on a combinatorial solid-phase library of peptide ligands synthesized on resin beads, allowing the concentration of low-abundance proteins while decreasing the concentration of high-abundance proteins. The approach has been applied for many different biological samples, including blood [64].

Degradomics

Human serum contains thousands of peptides that derive from endogenous proteolysis, collectively termed 'degradoma' [65]. Interestingly, when Villanueva and colleagues applied proteomic tools in an automated fashion to analyze blood samples from patients presenting with three different types of solid tumors (bladder, prostate or breast cancer), a few key peptides were recognized (the signature of the disease) [66]. These peptides appeared to be breakdown products of abundant proteins. The authors concluded that exoproteases activity,

superimposed to the *ex vivo* coagulation and complement degradation pathways, contributed to the generation of cancer type-specific serum peptides [67]. A study conducted by Marshall and colleagues also presented evidence for the existence of a mass spectral fingerprint in blood of patients presenting with myocardial infarction [68]. However, further studies are needed to explore the normal biological trash present in all biological fluids, to be able to differentiate protein degradation from disease-related signatures.

Protein separation: 2D electrophoresis

Various platforms with multidimensional technologies are currently used in proteomic research [64], and several approaches have been reviewed by Flisher and colleagues in a recent paper concerning urinary proteome analysis [69]. All steps may induce artificial modifications of the proteins within the sample. 2D electrophoresis has been largely used over the last 25 years and still seems to have a place in proteome analysis. However, the technique is associated with several changes of proteins during either sample processing or electrophoresis [70,71]. Spurious spots due to de-amidation of asparagine and glutamine residues may be detected. Carbamylation (related to the use of urea as a denaturant) and desulfuration (arising after prolonged electrophoresis) are also associated with modification of the 2D gel electrophoresis pattern. Reduction and alkylation of proteins may prevent the generation of such spurious spots. When 2D gel electrophoresis is used to evaluate the proteins content in various samples, gel-to-gel pattern variation is assumed to represent the biological difference between samples. In this sense, the application of 2D difference gel electrophoresis appears to be interesting for biomarker research, because different samples are analyzed within a single set of experiments [72,73]. To illustrate this approach, we applied this technique to study the influence of two variables (type of anticoagulant and number of freeze/thaw cycles) on electrophoretic patterns of plasma and serum prepared as illustrated in FIGURE 1. Electrophoretic methods used in this study have been described elsewhere [74,75]. Our data revealed that neither the choice of anticoagulant, nor the repeated freeze/thaw cycles, interfered with electrophoretic properties of abundant proteins. However, as expected, fibrinogen and prothrombin were only observed in plasma samples (FIGURE 2).

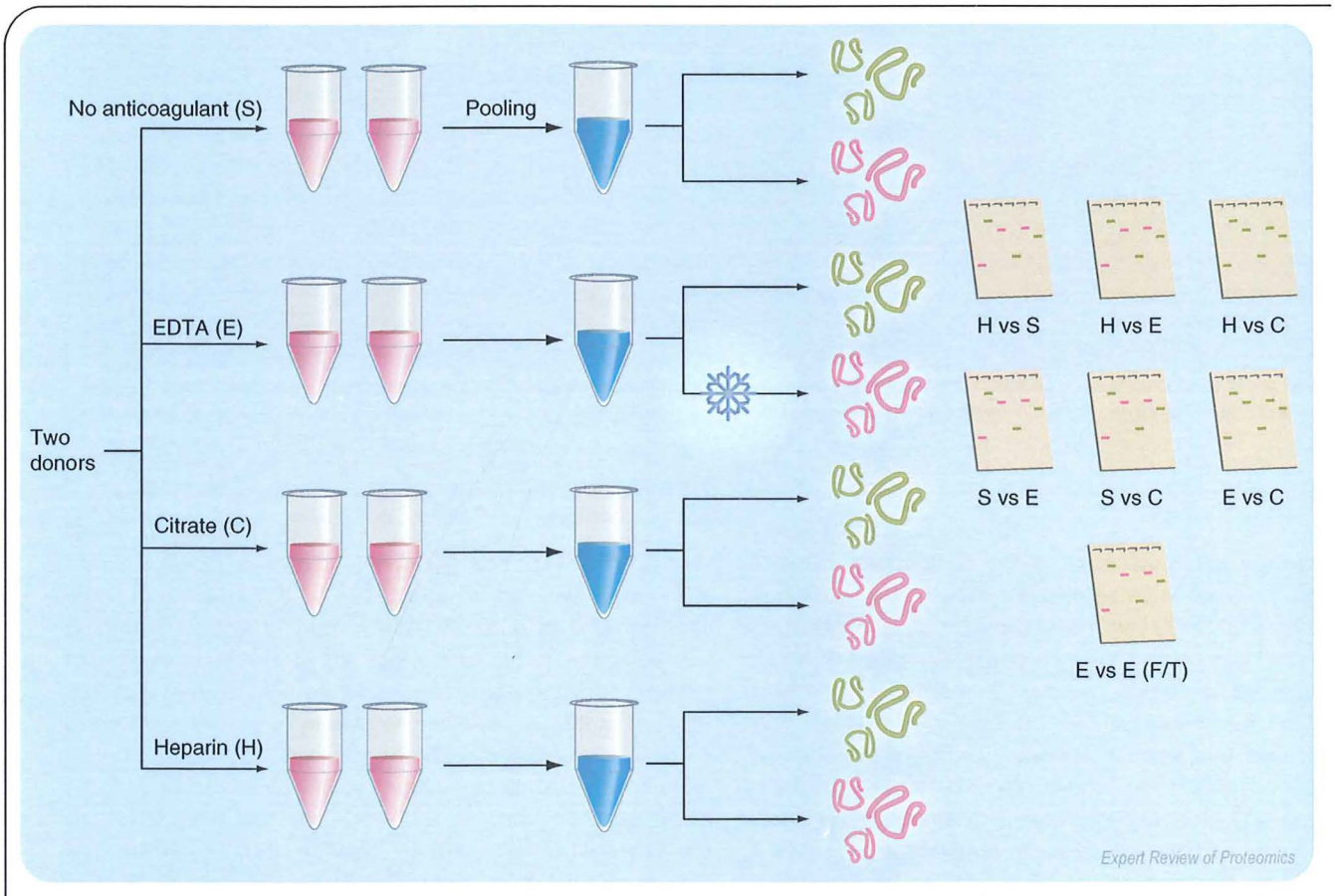

Figure 1. Flow chart describing handling procedures for plasma versus serum 2D electrophoresis analysis. After blood collection in BD Vacutainer® and Sarstedt Monovette® tubes, samples were each centrifuged at 2710 g at 15°C for 4 min. The resulting serum/plasma were pooled for each condition and were prepared for 2D difference gel electrophoresis [74,75]. The snowflake represents the freeze/thaw cycles. The green and pink bars represent the Cy3 and Cy5 staining, respectively.
Cy: Cyanine; F/T: Freeze/thaw.

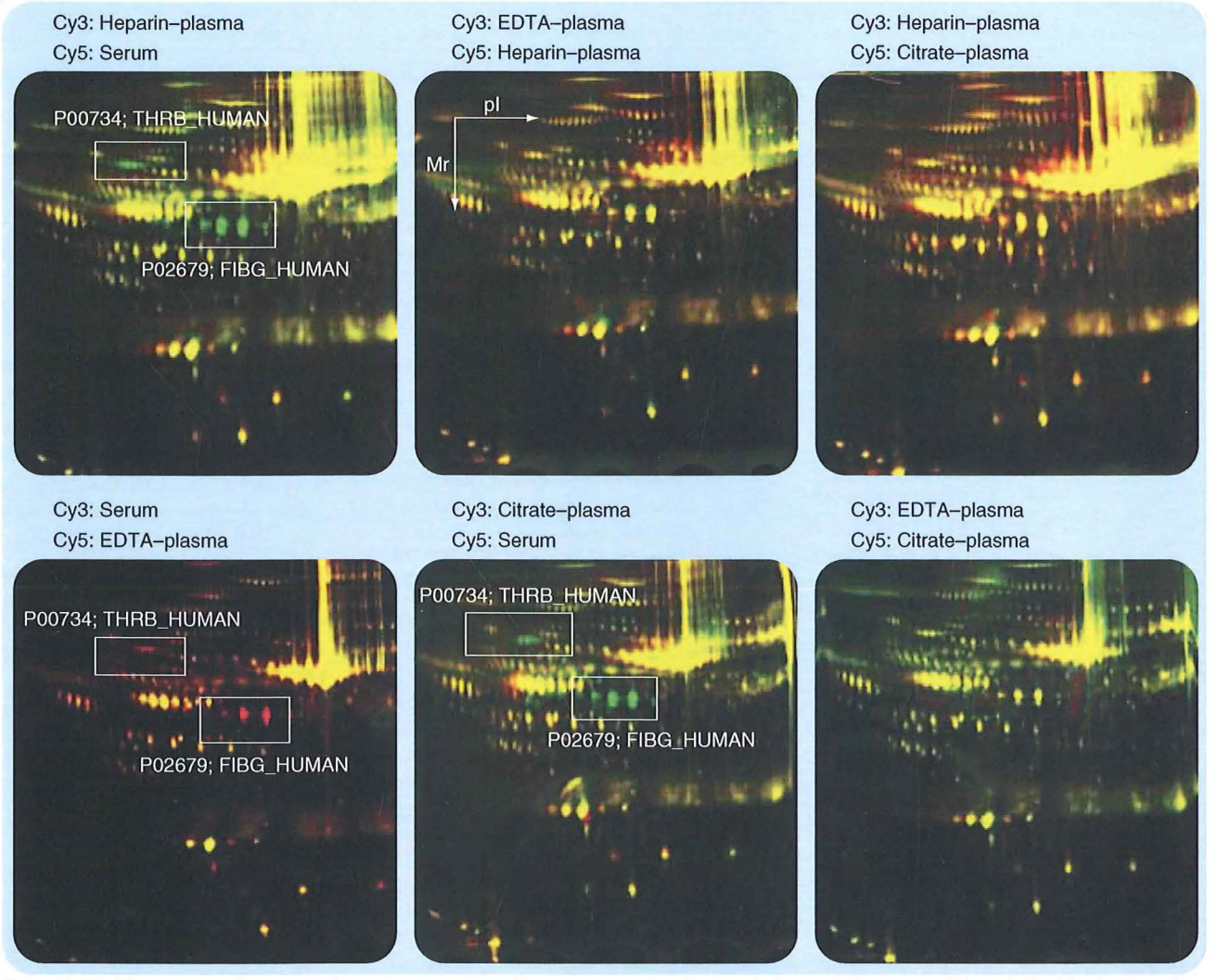

Figure 2. 2D difference gel electrophoresis patterns corresponding to serum and each type of plasma, following conditions defined in the flow chart.
First dimension: immobilized 4–7 pH gradient; second dimension: 9–16% polyacrylamide gel electrophoresis. White boxes indicate thrombin and fibrinogen with accession numbers corresponding to SwissProt database.
Cy: Cyanine; Mr: Molecular weight; pI: Isoelectric point.

Expert commentary

Omics sciences are rapidly developing and the search for validated biomarkers will be a challenge for the near future. The proteome analysis of human body fluids is in progress. The identification as well as the characterization of validated and clinically relevant biomarkers are still hampered by the presence of protease leading to protein degradation, as well as to high-abundance proteins that may adsorb several peptides of interest. A series of studies will be necessary to better define all pre-analytical steps that are critical for proteomic studies. With well-framed clinical questions, carefully selected clinical and control populations, appropriate numbers of samples in each group considered, perfectly controlled sample preparation and handling alongside accurate proteomic technologies, clinical proteomics will certainly emerge as a promising and valuable approach for scientific discoveries of at the beginning of the 21st Century.

Five-year view

With the extension of research and worldwide exportation of knowledge, patient care tends to be more complex, but more standardized (e.g., evidence-based medicine). The application of omic sciences will change many paradigms and clinicians are faced with a number of unsolved questions. All tissues and body fluids will be explored and are sources of biomarkers. Benefits resulting from proteomic studies, and more particularly from plasma/serum proteomics, will clearly depend on the quality management of all steps involved in the (pre-)analytical process. The automation of sample preparation, coupled with quantitative MS analyses, is becoming more and more available in many research laboratories through the world. The translation of the results of proteomics into clinical practice and patient care is still a dream, but who knows? In this context, the next 5 years will be a challenge for all scientists involved in clinical proteomics.

Key issues

- Performing proteome profile analysis necessitates optimization of two interdependent steps: pre-analytical and analytical phases.
- Handling procedures imply *per se* modifications of the sample, inducing artifactual results.
- Sample preparation must be conducted in a simple and well-defined sequence, thereby enabling detection and identification of uncontrolled variables.
- Reduction of the sample complexity, either by fractioning or by removal of high-abundance proteins, will certainly enable a more precise and accurate exploration of the plasma/serum proteome.
- Degradomics is rapidly developing and will help to separate biomarker discovery from the biological trash that is currently unexplored.

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REVIEW

Oxidation of proteins: Basic principles and perspectives for blood proteomics

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Protein oxidation mechanisms result in a wide array of modifications, from backbone cleavage or protein crosslinking to more subtle modifications such as side chain oxidations. Protein oxidation occurs as part of normal regulatory processes, as a defence mechanism against oxidative stress, or as a deleterious processes when antioxidant defences are overcome. Because blood is continually exposed to reactive oxygen and nitrogen species, blood proteomics should inherently adopt redox proteomic strategies. In this review, we recall the biochemical basis of protein oxidation, review the proteomic methodologies applied to analyse redox modifications, and highlight some physiological and *in vitro* responses to oxidative stress of various blood components.

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1 Introduction

Oxidative modifications to proteins have been for the most part considered as deleterious, irreversible, and ultimately leading to protein inactivation, degradation, and clearance [1, 2]. From decades of molecular studies of protein oxidation, the picture has dramatically evolved, and protein oxidations are now considered as two-faced modifications: on the one hand, oxidation mechanisms take part in many normal regulatory processes (beside energy conversion), such as enzyme activity modulation [3], signalling [4, 5], or gene regulation [6–9]. On the other hand, oxidative modifications also appear when oxidative stress overcomes antioxidant defences, and are then damaging [10, 11]. The response of living systems to oxidative stress is of primary importance in understanding cellular defence and aging [12–15]. Disorders of normal oxidative metabolism, or damages due to oxidative stress, have also been proven to be key players in a broad spectrum of diseases, from neurodegenerative disorders, such as Alzheimer disease [16–22], to various kinds of cancer [23–26], diabetes [27–29], and atherosclerosis [30–33].

From a molecular point of view, protein oxidation results in a wide variety of chemical modifications, ranging from protein backbone cleavage or protein crosslinking, to amino acid side chain subtle modifications. Moreover, oxidative damage can introduce new reactive chemical groups into proteins, such as aldehyde and ketones, leave nonconventional peptidic ends at both the N- and C-termini. Such an array of modifications is difficult to tackle with a single analytical approach, and large-scale studies of protein oxidations have usually focused on the detection of a single modification, such as cysteine or tyrosine oxidation, or a subclass of

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Abbreviations: DNPH, dinitrophenylhydrazine; **ECD,** electron capture dissociation; **MBLT,** methylene blue light treatment; **NO,** nitric oxide; **SOD,** superoxide dismutase

oxidation by-products, such as protein carbonyls. Whereas these approaches are compulsory steps on the way to understand oxidation processes and their effect on a large scale, there is no way to date to get a full picture of a proteome oxidative status.

Nevertheless some blood components are inherently subjected to oxidative stress. For example, RBCs are typically exposed to continuous fluxes of ROS due to their function; platelets are exposed to ROS at sites of inflammation, where coagulation happens. Additionally, protein oxidation mechanisms are of particular interest in transfusion medicine, and have been hypothesised to be responsible for the "blood storage lesion" [34–38]. Whether blood product oxidation is due to exposure of blood to oxidizing agents during puncture, handling, and blood product preparation (*e.g.*, pathogen inactivation procedures), or appears only during storage as a result of aging or stress is still unclear. In this review, we give a biochemical overview of protein oxidation processes, discuss the main methodological and instrumental approaches for the study of protein oxidation on a large scale, and present selected examples with relevance to blood analysis.

2 Biochemical overview

The gist of this section is not to give a comprehensive and detailed overview of protein oxidation mechanisms; this has been done elsewhere [39–44]. It is rather to provide an overview of protein oxidation products in terms of diversity and chemical specificity in order to highlight the possible analytical workflows and current challenges in redox proteomics. Oxidative modifications of proteins are due to attacks by ROS such as hydrogen peroxide (H_2O_2), anion superoxide (O_2^-), or hydroxyl radical ($OH^\bullet$), and reactive nitrogen species (RNS) such as nitric oxide (NO), nitrate (NO_3^-), nitrite (NO_2^-), and peroxinitrites ($ONOO^-$), as shown in Fig. 1 [45]. These species can appear as by-products of oxygen metabolism, or be present in the environment, and their appearance is the result of a complex interplay between the environment, and the cellular enzymatic machinery. The attack of proteins by these highly reactive species can lead to amino acid side chain modifications, cleavage of protein backbone, generation of carbonyl derivatives and formation of crosslinked protein complexes. Some reactions are limited and specific to certain residues, whereas others give rise to widespread and nonspecific modifications. Moreover, reactive oxygen and nitrogen species are also responsible for damages to DNA bases and sugar moieties, and degradation of lipids through peroxidation, the by-products of which can in turn modify proteins.

2.1 Protein backbone oxidation and cleavage

Protein backbone can be attacked by hydroxyl radicals on the α-carbon of amino acids, resulting in the formation of a carbon-centred radical. Under anaerobic conditions, two such

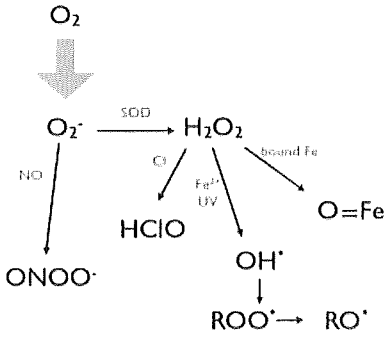

Figure 1. ROS and RNS. SOD stands for superoxide dismutase.

carbon-centred radicals can combine to form an intra- or interprotein crosslinkage (see below). In the presence of oxygen, a hydroxyl group can be added to this carbon-centred radical. The hydroxylated α-carbon can then undergo peptide backbone cleavage at the N–C bond through the α-amidation pathway, which leaves an amide at the C-terminal side of the N-terminal part of the protein, and a α-keto-acyl residue at the N-terminal side of the C-terminal part of the protein [40, 42, 46–48].

The same carbon-centred radical can undergo further attack by O_2, that induces cleavage of the peptide backbone, at the C–C bond through the diamide pathway, as shown in Fig. 2A. The diamide pathway initially induces one cleavage, leaving a diamide derivative on the C-terminal side of the N-terminal part of the protein, and an isocyanate derivative on the N-terminal of the C-terminal part of the protein, that spontaneously form the derivatives shown in Fig. 2A [40, 46, 47].

Additionally, oxidation of glutamyl and prolyl residues can also result in single backbone cleavage. As shown in Fig. 2B, the cleavage at glutamyl residue leaves an amide at the C-terminal side of the N-terminal part of the protein, and a pyruvyl residue at the N-terminal side of the C-terminal part of the protein, whereas the prolyl oxidation leaves two protein fragments with conventional termini and releases α-amino butyric acid [47].

Lastly, beta-scission can occur through radical attack on the β (C3) position, as shown in Fig. 2C [40, 42, 49]: the release of the side chain as a carbonyl compound leaves a radical on the α-carbon, which is then prone to backbone cleavage through mechanisms similar to that of the diamide or α-amidation pathways.

2.2 Protein carbonyls

Protein carbonyls appear through side-chain oxidation of proline, arginine, and lysine, as shown in Fig. 3 [13, 42, 50]. They can also result from backbone cleavage through the α-amidation pathway or β-scission. Alternatively, they can be introduced into proteins through Michael addition of unsaturated aldehydes produced by peroxidation of lipids (the

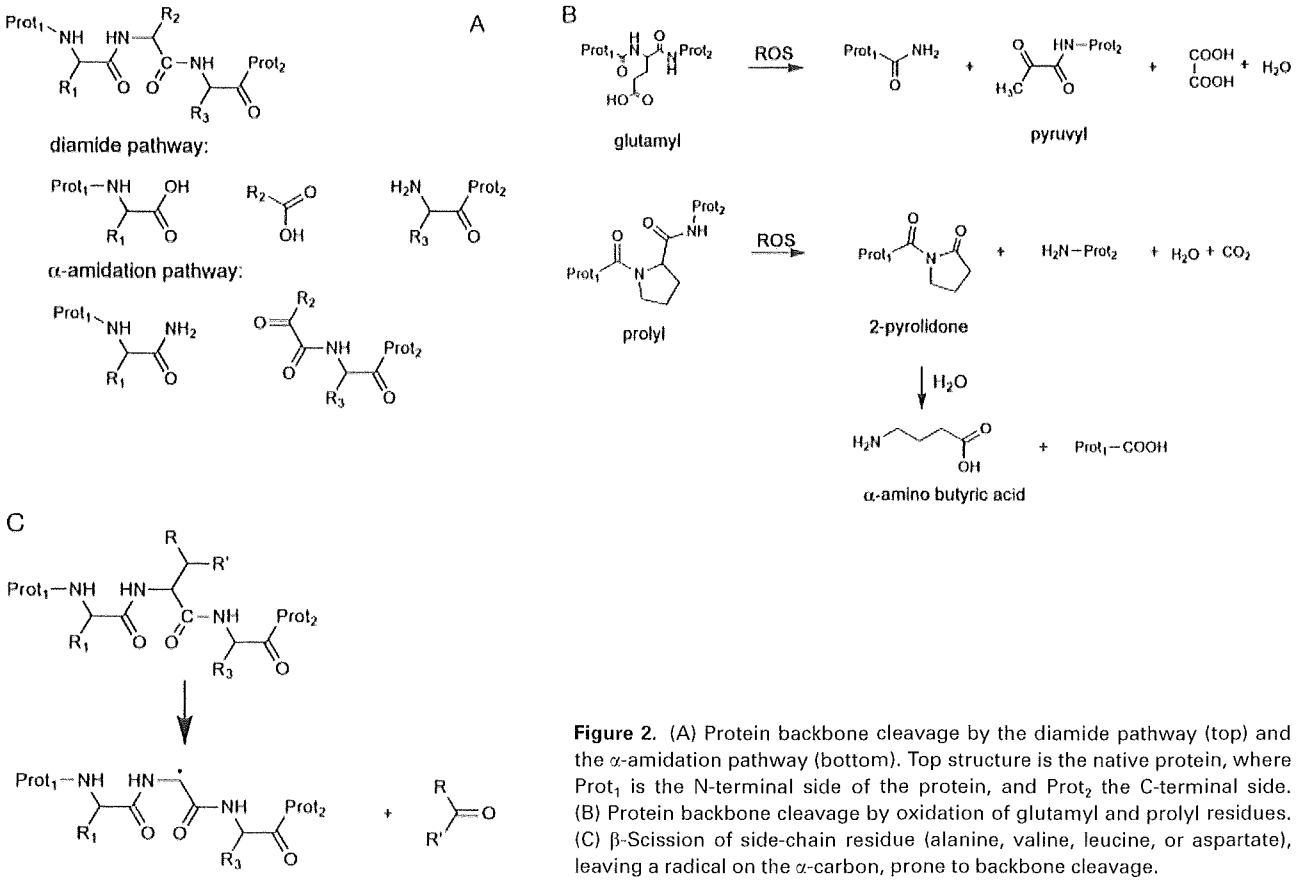

Figure 2. (A) Protein backbone cleavage by the diamide pathway (top) and the α-amidation pathway (bottom). Top structure is the native protein, where $Prot_1$ is the N-terminal side of the protein, and $Prot_2$ the C-terminal side. (B) Protein backbone cleavage by oxidation of glutamyl and prolyl residues. (C) β-Scission of side-chain residue (alanine, valine, leucine, or aspartate), leaving a radical on the α-carbon, prone to backbone cleavage.

main product being the addition of 4-hydoxy-2-nonenal on cysteine, histidine, and lysine, as shown in ref. [51–54] and in Fig. 4).

As carbonylation results in the introduction of reactive aldehyde or ketone groups in the protein, they are easily quantifiable (see below) and are indeed considered in practice as reliable markers of oxidative stress [55, 56].

2.3 Protein thiols and thioethers

Cysteinyl thiols can undergo a large array of oxidative modifications, depending on their accessibility in the protein structure, and the species they can contact to. Moreover, as cysteines play a pivotal role in protein structure through the formation of disulphide bonds, their oxidation status is of primary importance for protein function. In the recent years, cysteine oxidation has been more and more recognised as a basal regulation mechanism [57]. Free sulphydryl groups can undergo direct, reversible oxidation to sulphenic acid, and most often further irreversible oxidation to sulphinic and sulphonic acid, as shown in Fig. 5. Free cysteines can also be nitrosylated [58].

In addition, free sulphydryl groups can also form disulphide bridges with low molecular weight sulphydryl compounds present in the protein environment, such as free cysteine, and glutathione [59–62]. S-glutathionylation is in most cases a permanent modification, except if a second cysteine is present in the close vicinity, and is available for disulphide bridge formation.

Together with cysteine, methionine belongs to the most easily oxidisable amino acid. Its oxidation products are shown in Fig. 6 [63]. The cyclic oxidation–reduction of methionine through NADPH-dependant thioredoxin reductase is an important antioxidant mechanism [64–67]. Age-dependent increase in methionine sulphoxide content of proteins was reported for different tissues, notably erythrocytes [67].

2.4 Nitrotyrosine

Peroxynitrite ($ONOO^-$) results from the reaction of superoxide (O_2^-) with NO (see ref. [45, 68–77] for review). It is a strong oxidant with a short biological half-life. Once formed intravascularly, it can directly undergo oxidation with several biological targets or generate radicals resulting later in oxidation and nitration reactions. Tyrosine nitration occurs *via* a two-step mechanism: (i) a tyrosyl radical is formed, (ii) the tyrosyl radical reacts with the free radical NO to form 3-nitrotyrosine (Fig. 7) [78]. The latter has been revealed as a

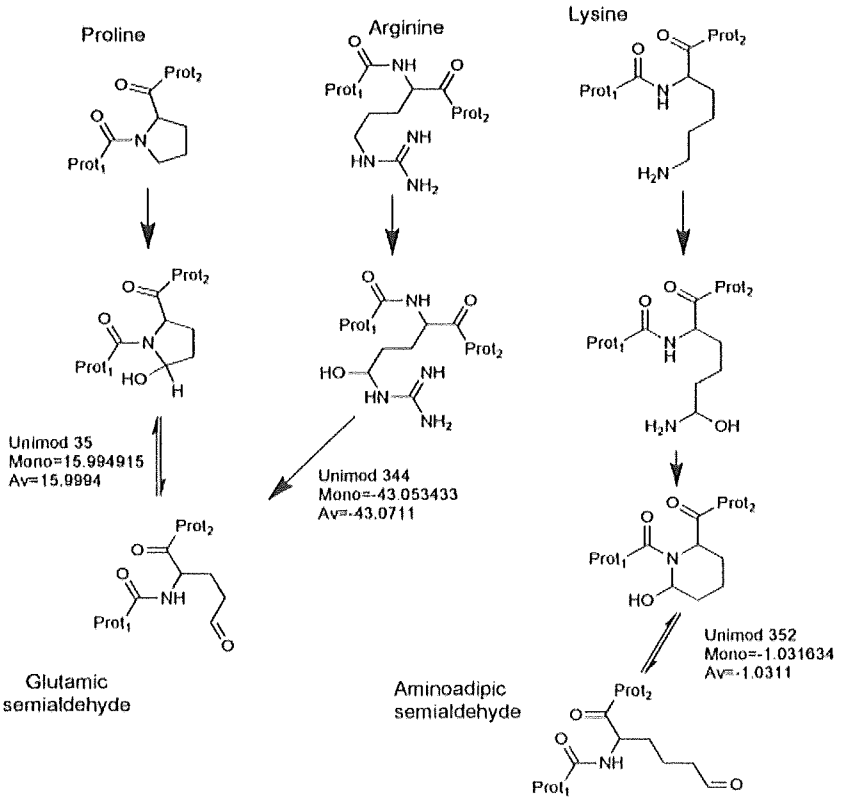

Figure 3. Carbonylation of amino acids side chains. Mass differences are given compared to the unmodified amino acid [196].

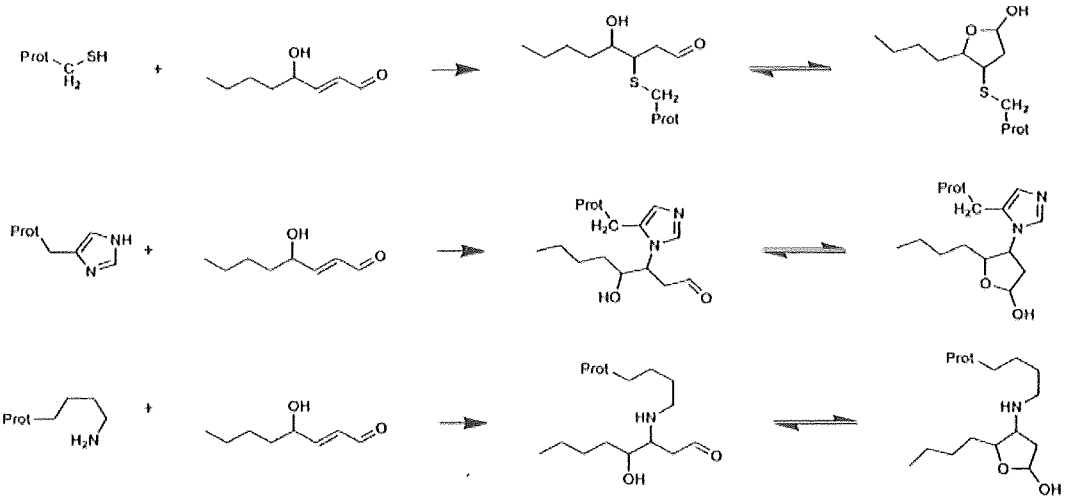

Figure 4. Introduction of carbonyls into proteins through Michael addition of 4-hydroxy-2-nonenal on cysteine (top), histidine (middle), and lysine (bottom) side chains.

biomarker of nitrosative stress and may serve as predictor of coronary artery disease [73, 79].

2.5 Protein crosslinking

As mentioned above, protein backbone can be attacked by hydroxyl radicals on the α-carbon of amino acids, resulting in the formation of a carbon-centred radical. In the absence of oxygen, two such carbon-centred radicals can combine to from a covalent intra- or interprotein crosslink. Additionally, intra- or interprotein crosslinks can appear through cysteine oxidation *via* the formation of disulphide bridges. Lastly, other crosslinks induced by oxidation of specific residues have been reported, such as dityrosine formation [80], or

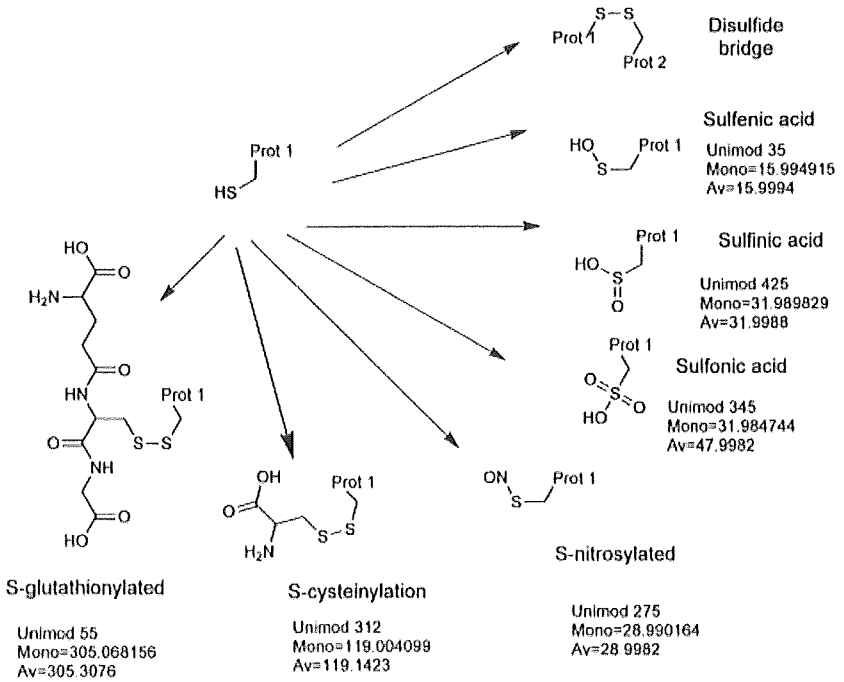

Figure 5. Main oxidation products of cysteine. Mass differences are given compared to the unmodified amino acid.

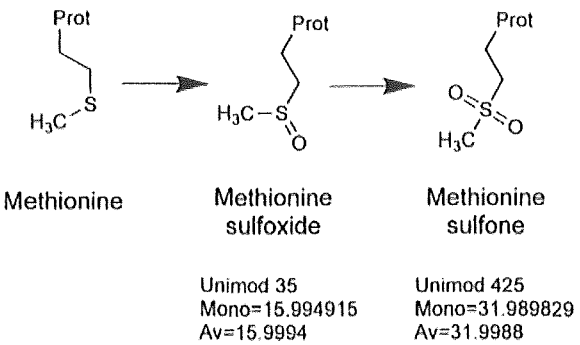

Figure 6. Methionine oxidation products.

sulphur–nitrogen crosslinking (for example, Cys–Lys and Cys–Arg) [81].

3 Methodologies for redox proteomics

The field of redox proteomics inherits a whole armada of methodologies for the analysis of protein oxidation products from classical biochemistry studies. Nevertheless, proteomics aim at analysing the whole proteinaceous content of a given sample, and identifying all modifications present down to the single amino acid level. At the same time, oxidative modifications are nonstoechiometric, and present a large diversity (protein fragments with both conventional and nonconventional termini, hydroxylated protein backbone, carbonyls, oxidised cysteines and methionines, nitrotyrosines, crosslinked proteins, just to name the main modifications). There is to date no proteomic workflow able to catch up with such a diversity. Most redox proteomic studies thus focus on a particular type of oxidative modification, a few of which are described below.

3.1 Carbonyl detection and quantification

Carbonyls have been long regarded as global markers of protein oxidation. Carbonyl tagging is relatively easy to perform due to the present of reactive aldehyde or ketone groups. These groups react quantitatively with hydrazine to form hydrazone. Carbonyls can thus be quantified spectrophotometrically by 2,4-dinitrophenylhydrazine (DNPH, the structure of which is shown in Fig. 8). The results are usually expressed in moles of carbonyls *per* gram of proteins [82, 83]. Such spectrophotometric assay is not exempt from biases, such as the presence of excess DNPH [84], or nonprotein carbonyls. Alternatively, ELISA assays have been developed for the quantitation of DNPH-derivatised carbonyls [85–87]. The same chemistry can be used in combination with gel electrophoresis, followed by an immunodetection [88, 89]. Alternatively, Yoo and Regnier [90] have developed a biotinylation strategy for the specific labelling of carbonylated proteins after 2-DE.

3.2 Carbonyl enrichment

Another possible strategy is to use DNPH derivatisation in combination with anti-DNPH antibodies to immunoprecipitate and enrich carbonylated proteins, which has been demonstrated by England and Cotter in the study of ER pro-

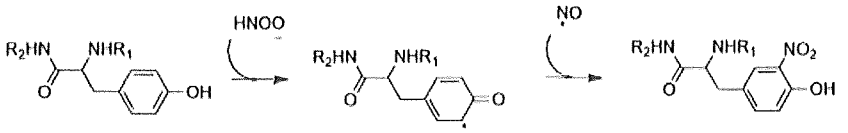

Figure 7. Formation of 3-nitrotyrosine.

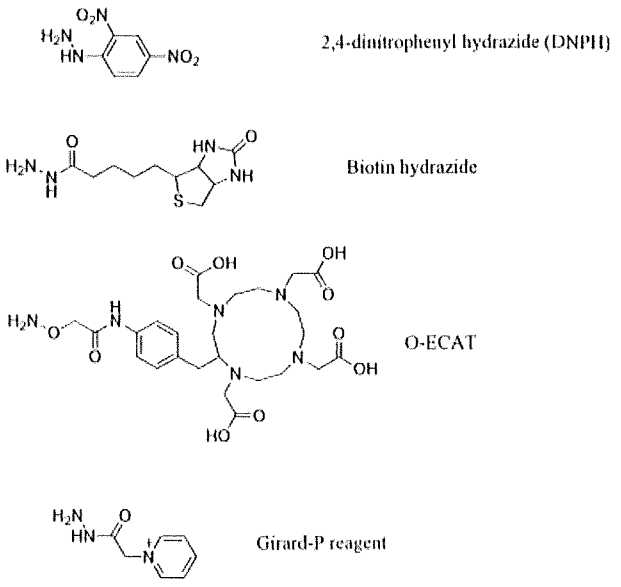

Figure 8. Probes reactive toward the aldehyde and ketone groups of carbonyls. On the O-ECAT reagent, the four nitrogen atoms can coordinate a metal centre (not shown here).

tein susceptibility to oxidation by 2-DE and MALDI-TOF MS [91], and by Kristensen *et al.* [92] in rice leaf mitochondria oxidation study with 2-D LC-MS/MS.

An alternative is to use affinity baits for the specific isolation of carbonylated proteins. For example, one can use biotin hydrazine for the derivatisation of ketones and aldehydes, and avidin columns for specific isolation of derivatised peptides and proteins [93–98]. Interestingly, Mirzaei and Regnier [97] compared three different strategies based on biotin hydrazine tagging of carbonyls, affinity selection, proteolysis, RP-HPLC, and MS, and found that performing the affinity selection and chromatography at the protein level before proteolysis and mass spectrometric protein identification, was more informative because working with intact protein allowed the detection of crosslinked or truncated proteins. Using a similar approach, Roe *et al.* [99] directly derivatised glass beads with a hydrazine group, allowing spin down isolation of carbonylated proteins.

Regnier's group also introduced a different tagging reagent for carbonyls: Girard's P reagent, which bears a hydrazine group, together with a permanent positive charge. Using this tagging reagent in combination with strong cation exchange chromatography, authors were able to enrich carbonylated peptides [100], and quantify them through isotopically labelled Girard's P reagents [101].

Lastly, Lee *et al.* recently introduced the so-called "Oxidation dependent element coded affinity tags" (O-ECAT), which are probes bearing one aminooxy group able to form a covalent bond with aldehydes or ketones, and one metal-chelator moiety. Antibodies against the metal-chelator moiety allow the affinity selection of derivatised peptides and proteins, and the probe can be loaded with various metals prior to any labelling of carbonyls in order to provide relative quantification information in MS [102].

3.3 Probing thiols oxidation

Methods for the study of protein thiols oxidation states have been the subject of recent reviews [44, 103]. Briefly, most techniques lie in the differential labelling of free sulphydryls and oxidised ones, for example, through maleimide, iodoacetamide, iodoacetate, and thiosulphate chemistries. For example, Baty *et al.* [104, 105] first blocked free cysteines with an alkylating reagent, then reduced the sample to make sulphydryls previously involved in disulphide bridges and gluthationylation available, and labelled them with a fluorescent probe before 2-DE. Authors were thus able to study the effect of oxidants (such as diamide or H_2O_2) on thiols oxidation state, and therefore identify proteins susceptible to oxidation. Similarly, Laragione [106, 107] used the same method with a probe containing an affinity bait (biotin), and further detected initially thiol-oxidised proteins with a streptavidin–peroxidase conjugate after Western blotting.

The same methodology can be applied for the specific enrichment of oxidised-thiol containing proteins: free thiols are first blocked, oxidised thiols are reduced chemically and further reacted with a probe containing an affinity bait, such as biotin [108–112]. When isotope-coded-affinity-tags (ICAT) reagents are used in this way, relative quantification between two differentially oxidised samples can be obtained [113, 114]. These techniques are useful to identify oxidation-sensitive thiols, but fail to identify the type of oxidation.

More specific is the probing of cysteines susceptible to Sglutathiolation: Brennan *et al.* mimicked a particular oxidative stress by adding biotin-GSSG-biotin to rat tissues; upon excess GSSG, disulphide exchange occurs and gluthationylated proteins can be isolated by avidin columns. Doing so, authors were able to study the proteome of thiols susceptible to S-gluthationylation in heart tissues [115, 116].

Jaffrey *et al.* [117] also targeted a specific cysteine modification (S-nitrosylation, see Fig. 5) by first blocking cysteines, reacting nitrosothiols with ascorbate to leave free

cysteines, and finally reacting these cysteines with biotin-containing reagents for specific enrichment of initially S-nitrosylated proteins.

3.4 Nitrotyrosine

Most studies of tyrosine nitration [118] rely on immunological detection thanks to commercially available anti-nitrotyrosine mAb, whether in the ELISA format [119], in which case it is difficult to distinguish between free circulating 3-nitrotyrosine and protein-bound nitrotyrosine, or in Western blot format after 1-D or 2-DE [120–122]. Immunoprecipitation with antinitrotyrosine antibodies allowed Nikov *et al.* [123] to map nitration sites of HSA by MS.

Interestingly, Zhang *et al.* [124] have recently introduced a methodology for selective isolation of nitrotyrosine-containing peptides: first, primary amines are acetylated, and free sulphydryls are blocked, nitrotyrosines are then reduced to aminotyrosine, which are then acetylthioacetylated, and the resulting group is deprotected to leave a free sulphydryl group, which in turn allows specific enrichment of peptides initially containing 3-nitrotyrosine. Doing so, authors were able to dramatically enrich peptides containing 3-nitrotyrosine compared to a direct mass spectrometric analysis.

3.5 Mass spectrometric and bioinformatics challenges

MS is now well established as a central identification technology in proteomics. Nevertheless, the identification and location of PTMs remains a challenge in routine analysis [125]. In the specific context of oxidative modifications, two major difficulties arise due to heterogeneity of possible modifications; first, whereas MS/MS is perfectly suited to the detection and identification of chemical modifications on amino acid side chains, the number of possible modifications (such as the different oxidation states of cysteine or methionine, the presence of nitrotyrosine, to name only the most standard ones) as well as the possibility of non specific peptide cleavages dramatically increase the search space when trying to match tandem mass spectra to peptide sequences in the queried database. This in turn increases the probability of false identification [126].

Even more complex are the cases of backbone cleavages and interprotein crosslinking: because in bottom-up strategies, sequence coverages of identified proteins are intrinsically low, it is very difficult to unambiguously identify a protein fragment or the site of crosslinking (see below for an example about erythrocyte membrane proteins).

Additionally, many oxidised products are chemically unstable, and form adducts with other compounds; for example, one product of the hydroxylation of tyrosine is 3,4-dihydroxyphenylalanine. The latter can be converted to orthoquinone through metal catalysis, and then further undergo Michael addition with a free cysteine [127]. Such nonconventional and unexpected modifications are virtually impossible to track down with the large-scale tools of MS and bioinformatics.

Another possible complication due to instrumental artefacts is the loss of side-chain modifications during tandem MS. It has been observed that labile modifications are readily lost during gas-phase fragmentation by CID [128]. One promising solution to this problem is the development of "softer" fragmentation techniques such as electron transfer dissociation (ETD) and electron capture dissociation (ECD). For example, Guan *et al.* [129] showed that CID of oxidised methionine containing peptides resulted in the loss of CH_3SOH whereas ECD allowed fragmentation of the peptide backbone while preserving the side-chain oxidation, thus allowing direct location of the oxidised methionine.

Recently, Zhao *et al.* [130] proposed a complete methodology for single protein oxidation mapping based on high resolution, high accuracy MS: they mimicked oxidation of P21Ras by *in vitro* incubation with peroxynitrite or GSSH, and analysed both tryptic digests (bottom-up protein MS) and whole proteins (top-down protein MS) by CID and ECD. They were not only able to map the oxidative modifications to the protein, but also compare the reactivity of the different sites susceptible to undergo oxidative modifications. But such studies are possible at the single protein level, and data interpretation is yet hardly amenable to automation for large-scale studies.

4 Perspectives for blood proteomics

4.1 Red blood cells

The RBC proteome has been the subject of extensive efforts, and more and more data accumulate through time [131–141], providing a high quality dictionary of red blood cell proteins. Red blood cells are inherently under continuous oxidative stress, as they pass the lungs once a minute; they contain high levels of O_2 and haemoglobin which auto-oxidises to produce O_2^- and H_2O_2. The heme group of haemoglobin can serve as a Fenton reagent to initiate free radical reactions [142]. Additionally, the RBC is often considered as a sink for oxidative species [143–145]: approximately 40% of intravascularly formed peroxynitrite diffuses into RBCs: the peroxinitrite anion crosses the membrane *via* band 3, a bicarbonate-chloride exchanger, whereas diffusion of peroxynitrous acid is passive [146, 147].

The forefront of antioxidant defences has been identified to be superoxide dismutase (SOD), glutathione peroxidase (Gpx), peroxiredoxins (Prdx), and catalase, four enzymes that are highly abundant in red blood cells, as shown in Fig. 9. Superoxide dismutase catalyses the reduction of superoxide to oxygen and hydrogen peroxide through its [Cu–Zn] centre. Glutathione peroxidase catalyses the reduction of hydrogen peroxide to water by the conversion of GSH to GSSG, which can be recycled back to glutathione by the NADPH-dependent glutathione reductase. Catalase directly reduces hydro-

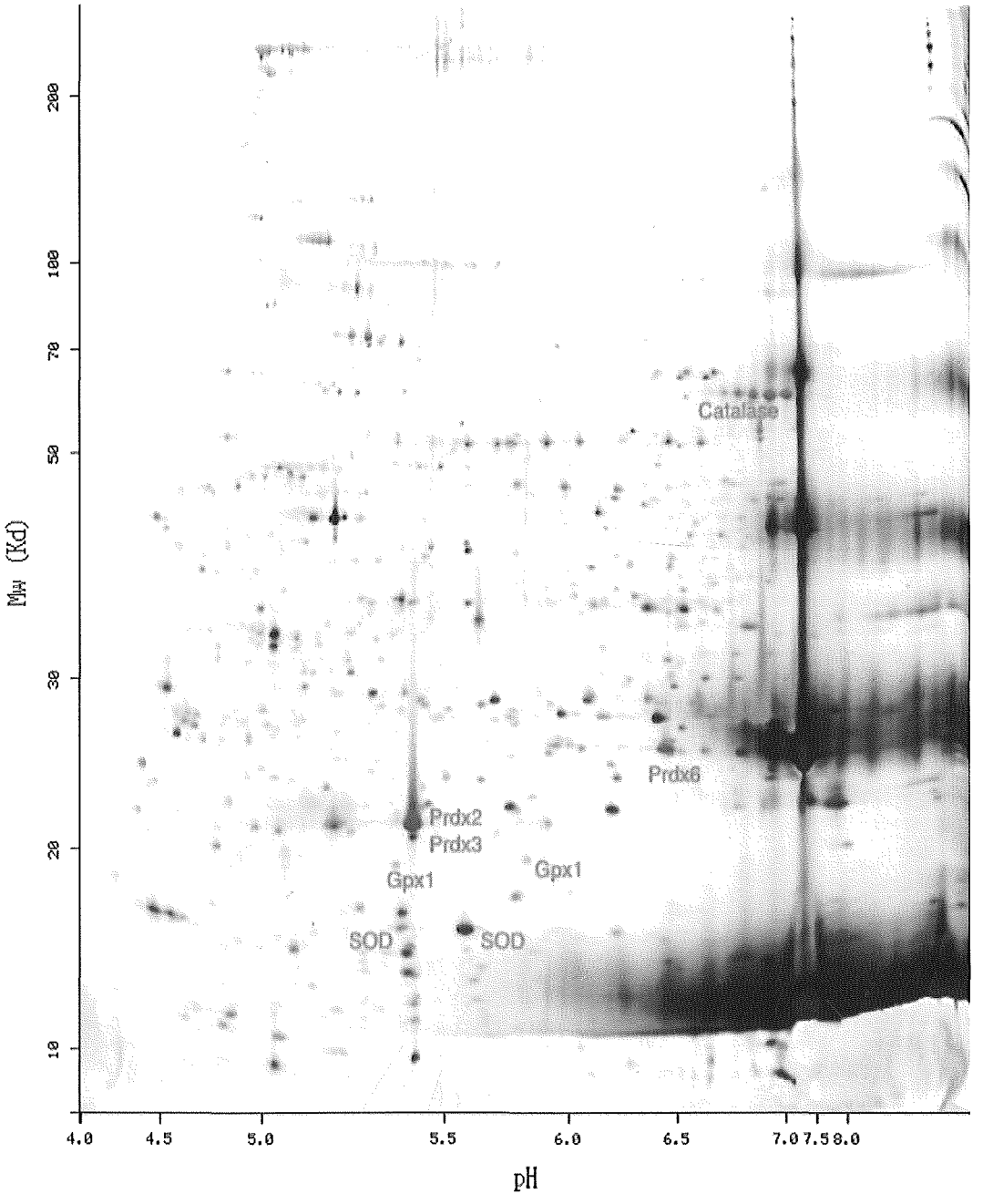

Figure 9. 2-DE of human red blood cells. Annotated in purple are proteins responsible for antioxidant defence (Prdx, peroxiredoxin; gpx, glutathione peroxidase; SOD, superoxide dismutase). The gel image was obtained from Swiss-2-D PAGE [197].

gen peroxide to water and oxygen in a two-step mechanism thanks to its heme group. Peroxiredoxins catalyse the reduction of H_2O_2 to water by oxidation of one cysteine (the peroxydatic one) to sulphenic acid; another cysteine (for the 2-cys peroxiredoxin subclass) reacts with the sulphenic acid to form a disulphide bridge. Its regeneration occurs through a thioredoxin /NADPH-dependent thioredoxin reductase system. The overoxidation of the peroxydatic cysteine may abolish the catalytic activity of peroxiredoxins [148].

The respective role of these enzymes in antioxidant defence is still a matter of debate. Knockout mouse models proved that the lack of peroxiredoxin I resulted in severe haemolytic anaemia, and appearance of lymphomas, sarcomas, and carcinomas [149]. The lack of peroxiredoxin II was also shown to result in haemolytic anaemia [150]. On the other hand, patients having hereditary catalase deficiencies were also shown to be victims of oxidative stress and presented a high prevalence of diabetes [151]. Gaetani *et al.* [152]

also demonstrated that catalase is essential for the removal of H_2O_2 from RBCs, having an activity six times higher than glutathione peroxidase. Peskin *et al.* [153] have recently shown that peroxiredoxin 2 and catalase react with H_2O_2 at comparable rates. It was hypothesised that catalase and peroxiredoxin play complementary roles in H_2O_2 detoxification/signalling due to the different recycling mechanisms used [154]; in RBCs, peroxiredoxin 2 was shown to accumulate as a dimer under H_2O_2 challenge, which was only slowly converted back to the active monomer by the thioredoxin system. This behaviour makes peroxiredoxin 2 ideally suited to H_2O_2 sensing at low concentration [155]. When H_2O_2 concentration increases, catalase and glutathione peroxidase are necessary to dispose of excess H_2O_2 [156]. What is unknown is the role of peroxiredoxin 2 dimer and overoxidised forms in H_2O_2 signalling.

Inside RBCs, oxidative stress induces haemoglobin crosslinking to the cytoskeleton [38, 157], which brings the heme centre in close vicinity to the phospholipid membrane where it can act as a local Fenton reagent [142]. Oxidative stress is also associated with protein degradation [158], band 3 clustering [159], phosphatidylserine externalisation [160], activation of caspases [160–162], and down-regulation of glycophorins [163], some of these being recognised as biomarkers of senescence or "apoptosis". In particular phosphatidylserine externalisation signals macrophages to recognise and degrade the RBCs.

Recently, D'Amici *et al.* [140] analysed by 2-DE the membrane proteome of RBC during storage, *i.e.*, during hypothesised oxidative stress. They were able to demonstrate dramatic alteration and cleavage of band 4.2, 4.1, band 3 and spectrin through the appearance of numerous spots through time, in addition to slighter modifications (hypothesised to be oxidative side-chain modifications) to numerous cytoskeleton, cytoskeleton-anchored and membrane proteins.

4.2 Platelets

Platelets are responsible for primary haemostasis through adhesion to collagen, release of mediators and aggregation with adjacent platelets. Platelets are exposed to ROS generated by the endothelial cells of the vessel walls; in addition, there is evidence that platelets can themselves produce ROS. Lastly, under inflammatory conditions, platelets are exposed the phagocyte-dependent, acute production of ROS [164].

Throughout haemostasis, the redox environment plays a critical role, notably with respect to platelet integrins. Platelet integrin αIIbβ3, a transmembrane fibrinogen receptor, is under tight regulation by sulphydril oxidation: the αIIb subunit contains 18 cysteines while the β3 subunit contains 56 cysteines. Part of them (located in the extracellular cysteine-rich domain of the β subunit) is present as free sulphydrils and remains available for redox regulation by extracellular factors. Additionally, the reduction of disulphide bridges appears to be involved in the conversion of αIIbβ3 to a fibrinogen-binding conformation, a phenomenon commonly referred to as "integrin activation". This modulation of αIIbβ3 integrin affinity for collagen is the result of an "inside-out" signal following platelet exposure to agonists or to adhesive subendothelial proteins. Fibrinogen binding, among other processes, is mediated by a protein disulphide isomerase (PDI) as well as ERP5, a thiol isomerase protein that is recruited to the cell surface during platelet activation. Similar mechanisms seems to account for other integrins, such as $\alpha_2\beta_1$ for example.

4.3 Other blood cells

Ghezzi *et al.* [165] identified two cysteines (52Cys and 62Cys) on cyclophilin A (CypA) as targets of glutathionylation in T lymphocytes and characterised the basis for the reactivity leading to this modification. Glutathionylation of these cysteines might interfere with the formation of the CypA/cyclosporin A complex or the binding of CypA with the HIV-1 capsid protein. It was shown that alterations in the antioxidant defence enzymes contributed to the outcome in diffuse large B-cell lymphoma, the patients with decreased manganese-SOD and thioredoxin inhibitor VDUP1 having the worst prognosis [166]. One explanation for this phenomenon is the modulation of glucocorticoid nuclear receptor function, which is redox sensitive [167].

Regarding blood stem cells, it has been shown that increasing levels of ROS act through defined mitogen-activated protein kinase pathways to limit the life span of cells *in vivo* [168].

4.4 Igs

The oxidation of antibodies increases the hydrophilic nature of the paratopes and increases their tendency to bind to cationic surfaces even without strong surface-to-surface fitting [169]. Studies on mAb during storage revealed clear sites of oxidation [170–172]. Recent findings suggest the existence of "redox-activated" autoantibodies that are not detectable by conventional immunoassays [173, 174]. A possible mechanism responsible for unmasking them may requires nitrosylation of tyrosine residues in the hypervariable or complementary determining region of Ig [175]. This concept has potential consequences in the understanding of the antiphospholipid antibodies syndrome, the opsonisation of aging erythrocytes and of their immune elimination [176].

4.5 Fibrinogen

Fibrinogen is a high abundant plasma protein and the major plasma coagulation factor. It consists of two sets of three disulphide-bridged chains (Aα, Bβ, and γ) of 610, 461, and 411 amino acid residues, respectively. Structure, heterogeneity, function and assays were all reviewed else-

where [177–180]. Western blot immunoassay showed that fibrinogen, among other plasma proteins, is highly susceptible to attack by oxidants [181]. Oxidant-induced carbonyl formation in fibrinogen derives largely from amino acid oxidation and not from oxidation of carbohydrate groups [182].

Previous experiments showed that histidine and tryptophan residues in the amino-terminal disulphide knot were affected by methylene blue light treatment (MBLT), which is a photosensitiser used for virus inactivation [183]. Photooxidation of an histidine in the Bβ-chain (16His) located only one amino acid residue away from the thrombin-susceptible bond was shown to impair fibrin polymerisation [184]. Addition of L-histidine, a target of singlet molecular oxygen generated during MBLT, was able to protect fibrinogen from the polymerisation defect in a dose-dependent manner [185]. Measurements of both the release of fibrinopeptide (by HPLC) and the generation of fibrin monomers (by electrophoresis) confirmed that oxidation-induced inhibition of clotting activity derived from an effect on fibrin monomer polymerisation, not from inhibition of thrombin activity [186]. S-nitrosothiols can induce changes in fibrinogen structure by interacting at specific domains rich in aromatic amino acids [187].

Oxidatively modified fibrinogen was also found to modulate blood rheological parameters [188]. Clinical implications of such results need obviously to be investigated further. Oxidised forms of fibrinogen circulating in blood could be interesting in several aspects, for example, for monitoring oxidative stress, controlling coagulation processes and studying protein senescence mechanisms.

4.6 Photoinactivation of blood products

Photoinactivation of blood products involves the addition of an exogenous agent or physicochemical manipulations: its benefits (the inactivation of pathogens) need to be balanced against deleterious effects on cells and plasma proteins [189]. UV irradiation has been proposed for pathogen inactivation of purified plasma proteins and clotting agents used in transfusion medicine. However it has been known for long that UV light exposure can damage proteins through generation of ROS, which can in turn damage proteins [190, 191]. Using 2-D DIGE and MALDI-TOF-MS, Chan *et al.* [192] identified alterations in protein thiol reactivity, indicative of an oxidative damage. Authors showed modification of various proteins involved in the coagulation cascade, such as kininogen, thrombin, albumin, actin, complement factor 4, serum amyloid P, or retinol binding protein.

MBLT stands as another option for pathogen inactivation of fresh frozen plasma and was also evaluated in the same terms [185, 193–195]. Figure 10 shows modifications of the 2-D pattern of fibrinogen γ chain, transthyretin, and apolipoprotein A–I upon prolonged light exposure in the presence of various concentrations of methylene blue.

5 Conclusion

In this review, we have tried to highlight the complexities of oxidative modifications to proteins, be it part of regulatory processes, responses to oxidative stress or permanent damages induced by exogenous compounds. As far as blood components are concerned, the selected examples described below show that there are complex processes to detect oxidative stress, and eliminate reactive oxygen and nitrogen species. In parallel, reactive oxygen and nitrogen species play physiological roles, such as the modulation of platelet activation for example.

Any blood proteomic approach should thus reveal the presence of oxidised proteins, due to physiological modifications (as is the case in RBC metabolism and platelet activation), and depending on how the sample was punctured, processed, and stored. The fact that most blood proteomic studies do not report such modifications just reflect the difficulty of analysing oxidative modifications to proteins on a large scale, mainly due to their diversity. Targeted strategies to detect and quantify oxidative modifications in blood components exposed various conditions (be it instrumental such as puncture and storage parameters), physiological or biomedical (samples from healthy individuals or with specific diseases) would be highly desirable and perfectly timely to increase our knowledge of blood physiology, give a sound basis for the search of biomarkers in plasma or other blood components, and practical recommendations for the handling, preparation and storage of blood products.

The authors have declared no conflict of interest.

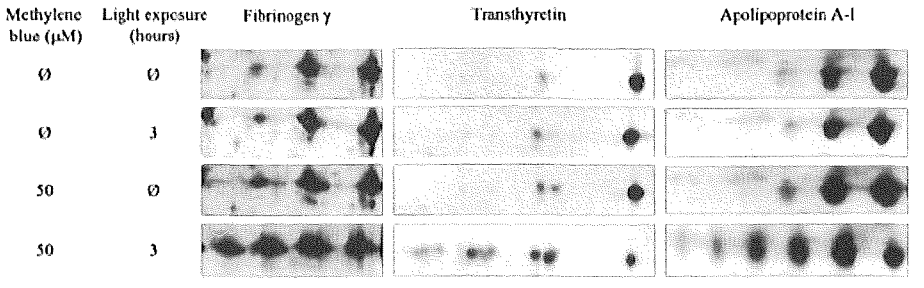

Figure 10. 2-D pattern of fibrinogen γ chain, transthyretin, and apolipoprotein A–I upon prolonged light exposure in the presence of various concentrations of methylene blue. Reproduced from [194] with permission.

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OXYDATION DU FIBRINOGENE HUMAIN PURIFIE PAR H_2O_2 : UNE ANALYSE PROTEOMIQUE PAR ELECTROPHORESE ET SPECTROMETRIE DE MASSE

OXIDATION OF HUMAN PURIFIED FIBRINOGEN BY H_2O_2 : A PROTEOMIC ANALYSIS BY ELECTROPHORESIS AND MASS SPECTROMETRY

RESUME

Les conditions de conservation du sang destiné à la transfusion créent un environnement par lequel l'oxydation aboutit à des altérations au niveau des protéines cellulaires et des facteurs de la coagulation. Le but du présent travail est de modéliser ces altérations en caractérisant au niveau moléculaire l'effet *in vitro* d'un traitement oxydant (eau oxygénée - H_2O_2) sur un facteur de la coagulation, le fibrinogène humain. La méthodologie se résume en quatre phases : 1) le traitement oxydatif de la protéine purifiée; 2) la séparation des sous-unités protéiques par électrophorèse bi-dimensionnelle ou par chromatographie liquide; 3) la digestion des protéines par la trypsine et l'analyse des peptides par spectrométrie de masse; 4) l'identification des peptides et la détection de modifications évocatrices d'oxydation. Les résultats indiquent au niveau de l'électrophorèse un changement du point iso-électrique, celui correspondant à la protéine oxydée étant plus acide que celui correspondant à la protéine native non-traitée. Aucun changement de masse n'est observé sur le gel d'électrophorèse. Au niveau du spectre de masse, nous pouvons distinguer plusieurs types de modifications selon que celles-ci sont ou non proportionnelles au traitement oxydatif. Les résidus oxydés sont principalement des méthionines avec une distribution relativement homogène au niveau de leur position moléculaire. Une part des modifications observées par spectrométrie de masse peut être attribuée à la phase pré-analytique. La conclusion de ce travail est que la spectrométrie de masse permet de localiser précisément les sites des résidus oxydés (*mapping*) et constitue en ce sens une approche complémentaire à l'électrophorèse bi-dimensionnelle. L'étude de l'impact de ces modifications sur la fonction du fibrinogène comme son interaction avec la thrombine et avec les glycoprotéines plaquettaires ou sa polymérisation en fibrine, offre une perspective intéressante à ce travail.

INTRODUCTION

Oxidative modifications on proteins are due to attack of reactive oxygen species (ROS). They can be considered in diverse frames. They can either be part of physiological processes such as enzyme activity modulation, signaling and gene regulation ; or they can reflect oxidative stress encountered in inflammation, cancer, degenerative disease and age-related changes[1;2]. In particular instances, and especially for blood proteins, oxidative modifications are due to preanalytical influences[3]. Oxidants such as peroxynitrite induces red blood cells damage, increasing the appearance of senescence markers such as thiol-oxidized skeletal proteins (spectrin) and rearrangement of membrane protein (band 3 and glycophorin A)[4].

Processing of blood products in transfusion medicine is hypothetically responsible for « blood storage lesions » via protein oxidation mechanisms[5-9]. Addition of methylene blue (MB) in plasma is able to inactivate viruses upon exposure to light[10-12]. Disadvantages of using MB-treated plasma are related to decreased levels of coagulations factors[13-18]. In order to gain insight in the quality of plasma obtained after MB light treatment (MBLT) and MB removal by filtration, Crettaz *et al* applied proteomics tools to characterize potential « injuries » on proteins[19]. A small-scaled analysis of 2D-GE gels (restricted isoelectric point (p*I*) range from 4.5 to 6.5 and low *M*r from 7 to 58 kDa) revealed 4 out of 387 matched spots that were modified by MBLT. These spots correspond to the gamma-chain of fibrinogen, apo-lipoprotein A-1 and transthyretin. According author opinion, the modification observed on 2D-GE (p*I* acidic shift) likely reflect imbalance of oxido-reduction of particular amino-acid residues rather than neo-antigen formation.

Our work aims to confirm this hypothesis with a similar experimental setting. We treated human purified fibrinogen with hydrogen peroxide (H_2O_2), and performed both 2D-GE and HPLC-MS/MS analysis.

Fibirinogen is the major plasma coagulation factor (1.5-4.5 g/l) and consists of two sets of 3 disulphide-bridged chains (Aα, Bβ and γ) of 610, 461 and 411 amino-acid residues, respectively. Details about structure, heterogeneity, function and assays are all reviewed elsewhere[20-25]. Fibrinogen plays a key role in haemostasis : following cleavage by thrombin, it undergoes polymerization to form an insoluble fibrin clot stabilizing the platelet plug. After release of fibrinopeptides, "knobs" are exposed at the centre of the molecule (E-domain) and fit into "holes" on neighbouring molecules (D-domain). This "knob-hole" interaction has been largely studied and modeled[26-33]. The globular carboxyl-terminal region of the γ-chain participates in many physiological interactions with other molecules. The ability of γ.chain to bind calcium and to interact with adjacent fibrin monomers or with the GPIIb/IIIa platelet receptor are the main determinants of fibrinogen functionality[34]. Some residues (histidine and tryptophan) of the amino-terminal disulphide knot are shown to be oxidized by MBLT[35]; L-histidine, a target of singlet molecular oxygen, quenches this reaction[36].

MATERIEL & METHODS

Oxidative treatment with H_2O_2

Human purified fibrinogen (plasminogen free) is reconstituted with distilled water to reach a 10% dilution (w/v) following instruction of supplier (Endotell, Allschwil, Switzerland). It is then transferred in 100 µl aliquots to be frozen (-80°C). Thawed aliquots are mixed with oxidants at different H_2O_2-concentrations, obtained from H_2O_2 30% w/v stock solution (Sigma-Aldrich, Buch Switzerland) diluted in distilled water. For 2D-GE analysis, a final H_2O_2 concentration of 5% or 10% is employed in contrast to that employed for LC-MS/MS analysis (0.1% and 1%). Oxidized samples are frozen at –80°C before use.

2D-GE protein separation

Sample for 2D-GE is prepared following a protocol adapted from the method described by Hochstrasser *et al*[37]. The addition of a SDS-DTE solution ensures a maximal dissolution of proteins in solution. After a short heating time (5 min at 95°C), protein extracts corresponding to 250 µg are rehydrated with a solution containing DTE, CHAPS, urea and ampholytes 3-10 (Pharmalyte, GE Healthcare, Glattbrugg, Switzerland) in order to obtain a final volume of 350 µl. Strips used for IEF (Immobiline Dry-Strip, pH range 4-7 or 3-10, 18 cm, GE Healthcare, Glattbrugg, Switzerland) is rehydrated in gel with the same solution. IEF is performed under paraffin oil, applying an increasing voltage for a total of 100kVh. Before performing the second dimension, strips are equilibrated with a solution containing urea, Tris-HCl, glycerol, SDS, DTE and iodoacetamide. Bromophenol blue is used as tracking dye. Strips are placed on the top of 9-16% gradient polyacrylamide gels that are copolymerized with piperazine diacrylamide as a cross-linker. Second-dimensional migration is performed with a current of 40 mA/gel. Gels are stained with silver according to the standard protocol.

LC-MS

Samples for LC-MS (20 µl) are thawed and lyophilized completely. They are re-suspended in 10 µl urea 8 M. The addition of 1 µl DTT 100mM ensures a good dissolution. Samples are heated at 50°C for 45 min and then cooled down at RT. After addition of 3 µl iodoacetamide 100 mM, they are put in the dark for 45 min. 1 µl DTT 100 mM and 70 µl ammonium bicarbonate 25 mM freshly made are added to the sample and pH adjusted to 7,5-8. In-solution proteolytic cleavage is made with 5 µg trypsin overnight and the reaction is quenched by the addition of 1µl TFA.

Mass spectra analysis

MSight software displays mass peaks obtained from MS. Differential-display patterns represent the mass (x axis), the time of elution (y axis) and peak intensity (intensity of spot).

An example of such pattern is shown in figure 1. Patterns are compared manually, with a particular attention on peptides whose mass shows a deviation of a multiple of 8 Da (assuming that peptides are 2+ charged). Charge state deconvolution and deisotoping are not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK). Mascot was set up to search the concatenated SwissProt and TrEMBL databases (selected for Homo sapiens) assuming the digestion enzyme trypsin, a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 50 ppm. Oxidation of cysteine, methionine, histidine and tryptophane is specified in Mascot as a variable modification. Subsequent MS/MS analysis specifically targets oxidized residues.

RESULTS

2D-GE pattern

The patterns of protein separation are shown in figure 2. The range of pH and orientation of gels are specified. In samples treated with H_2O_2, spots appear at a more "acidic" p*I* than that of control samples. Spots at the "basic" extremity of each chain lose their intensity. This acidic "shift" is not associated with an observable change in the mass of the protein.

MS-detection of oxidized residues

Peptides identification reveals sequence coverage of 32% for the alpha-chain, 55% for the beta-chain and 48% for the gamma-chain of human fibrinogen. Oxidation is detected on 26 residues, predominantly on methionines. Overall, comparing control (CTRL) samples with H_2O_2-treated samples, four categories can be distinguished among oxidation sites (See Table 1): in category I and II, the signals of oxidized peptides increase compared to control. In category III, the signals of oxidized peptides remain constant irrespective of the concentration of H_2O_2. In category IV, signals of oxidized residues are observed, but do not allow to conclude on their increase upon H_2O_2 treatment.

DISCUSSION

2D-GE gels show a change of p*I* toward acidity after oxidative treatment. This means a change of pKa constant. Theoretical titration curves show a similar change of pKa when cysteines of the gamma chain (pKa=8.4) are oxidized to sulfenic acids (pKa=4.5) and methionines are oxidized to methionine sulfoxides (pKa=4.6) (see figure 3), indicating that acidic shift is possibly due to residue oxidation by H_2O_2. Whereas the calculation is performed with grand averages of pKa values that do not reflect local microenvironment of particular amino acid side chains, the global behaviour is consistent with the patterns observed on 2D-GE (see Figure 2), where total trains of spots are shifted toward the acidic side upon oxidative treatment, and spots within the same train get sharper and closer from the basic side to the acidic side of the train.

MS analysis of peptides brings further results: 15 residues are found more oxidized (di-, tri-oxidation) in samples treated with H_2O_2 than those of control sample (category I). The fact that methionine is the predominant residue to be oxidized is not surprising[38]. Beta-chain of fibrinogen shows the maximum number of oxidation "events". This cannot be correlated with its length (shorter than alpha-chain and longer than gamma-chain). A possible explanation is a different access to oxidant for each chain in native form. Oxidations are distributed quite uniformly along the chains. There is no observed oxidation near the amino-terminal disulphide knot (E-domain).

Methionine in position 536 on alpha-chain for example (category I) is never found in its reduced form, indicating that oxidation probably would have occurred *in vivo* or during purification process. In this precise situation, we consider MS as highly indicative of pre-analytical "fingerprint". For category II, the fact that residues appear in a reduced form in control sample raises the question of bias due to our methodology: the DTT used before alkylation could reduced some cysteines that are reversibly oxidized by H_2O_2. An alternative

protocol would be to alkylate reduced cysteines (hence, cysteines that do not react with H_2O_2) before reducing them with DTT. Another alkylating agent (such as N-ethylmaleimide, for example) then is used to "lock" reduced cysteines that undergo reversible H_2O_2-oxidation specifically. Another bias regards the method of MS-spectra analysis. We focus on oxidative side-chain modifications and look for mass changes corresponding to a multiple of 8 Da (see methods); modifications such as backbone cleavage or cross-linkage are missed by such analysis.

CONCLUSION

The combination of 2D-GE and MS analysis proved to be useful for the assessment of oxidative damage to fibrinogen. On one part 2D-GE allows the global detection of oxidative damage by shifts in electrophoretic patterns of the different chains of fibrinogen. On the other part, MS analysis allows precise mapping and categorization of oxidation sites. However, no specific oxidative locus is identified in our study. Oxidation sites seem to be randomly distributed along the various fibrinogen chains, and no clear assignment of oxidative lesions to functional sites of the fibrinogen chains can be made.

PERSPECTIVES

Design of knobe-hole interaction by *in silico* tools could be performed on control and oxidized sample in order to correlate structure modifications with function. Protein such as fibrinogen is ideal for functional studies. Early reports indicate that oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation, due to impaired fibrin monomer polymerization rather than altered thrombin-catalyzed fibrinopeptide release[39;40]. Platelets adhesion and spreading assays could also be performed with oxidized fibrinogen in order to study its interaction with αIIbβ3 integrin[41;42].

Figure 1.

Differential-display patterns of LC-MS peptides

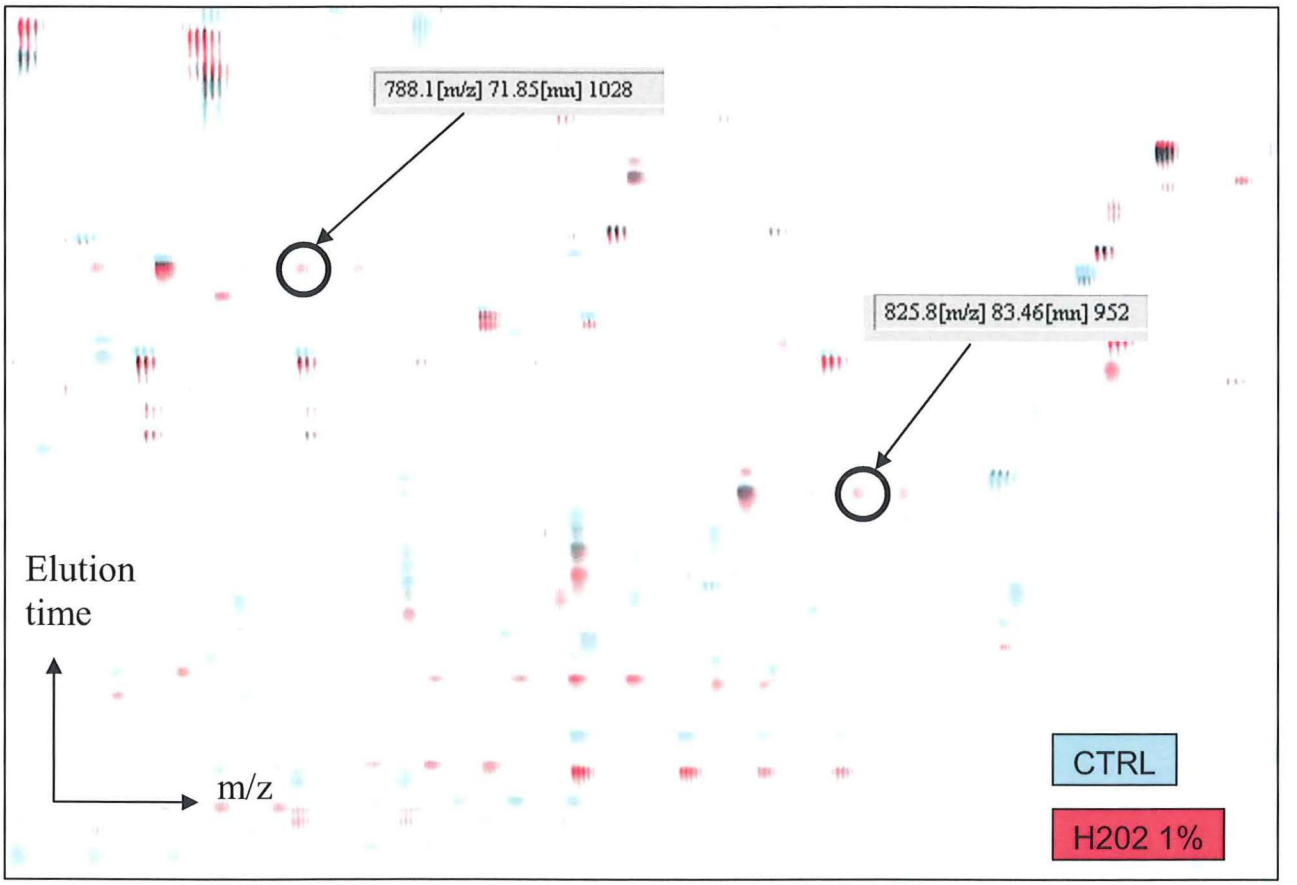


Figure 2.

2D-GE pattern of purified fibrinogen upon *in vitro* oxidation. On strips 4-7, only chains beta (more basic) and gamma are displayed.

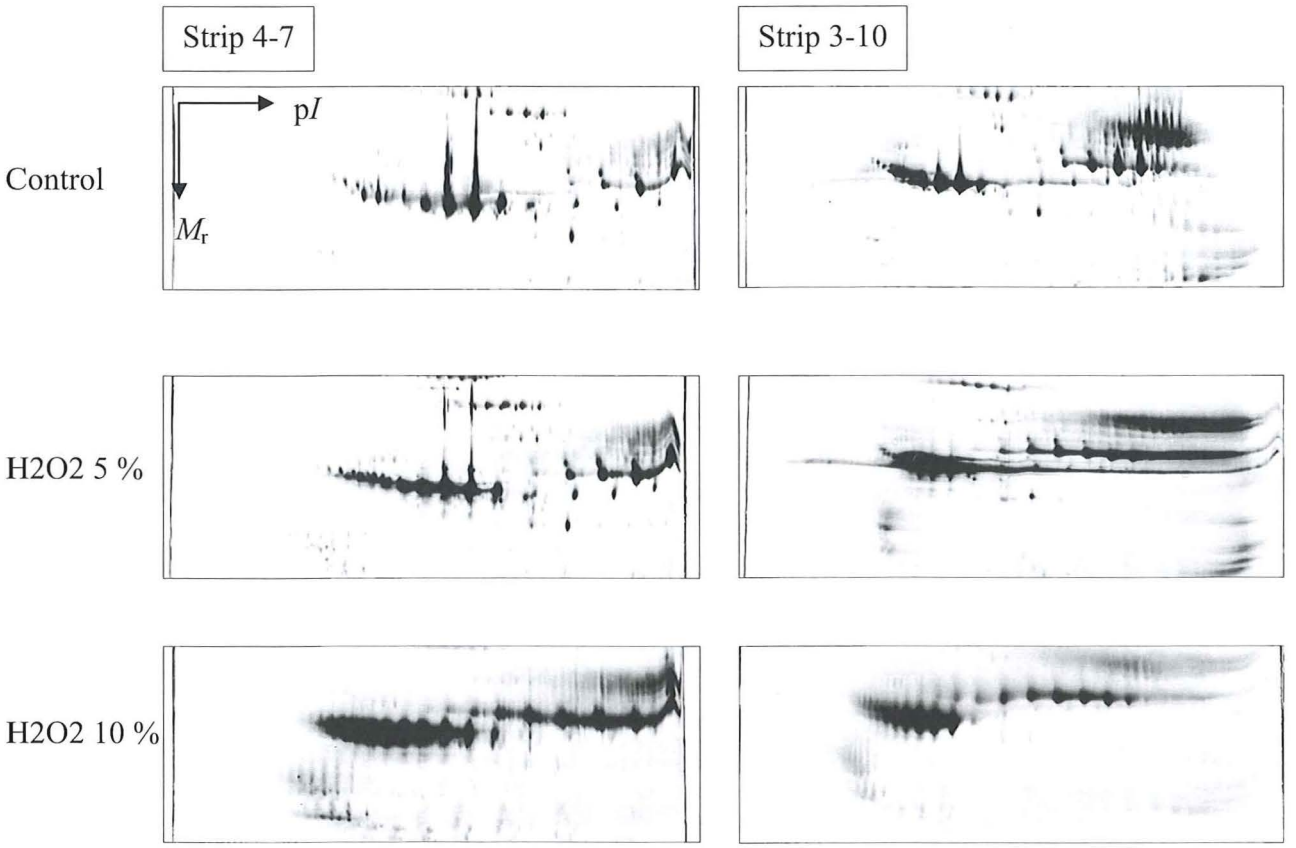


Figure 3.

Evolution of the titration curves (top) and theoretical spot positions (bottom) of fibrinogen gamma chain upon the oxidation of 0 to 9 cysteines to sulfenic acids and additional oxidation of 9 methionines to methionine sulfoxides.

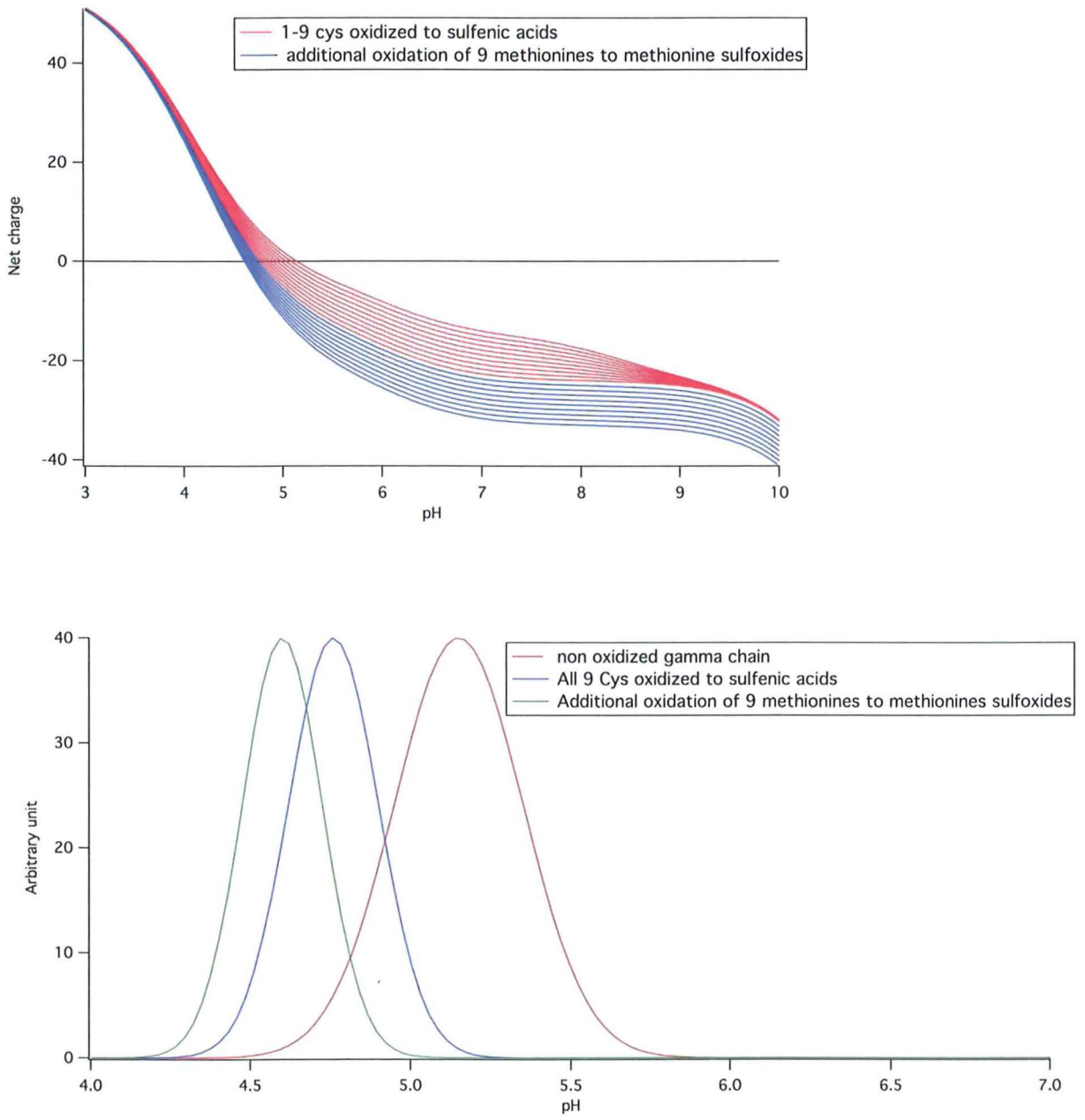


Table 1.

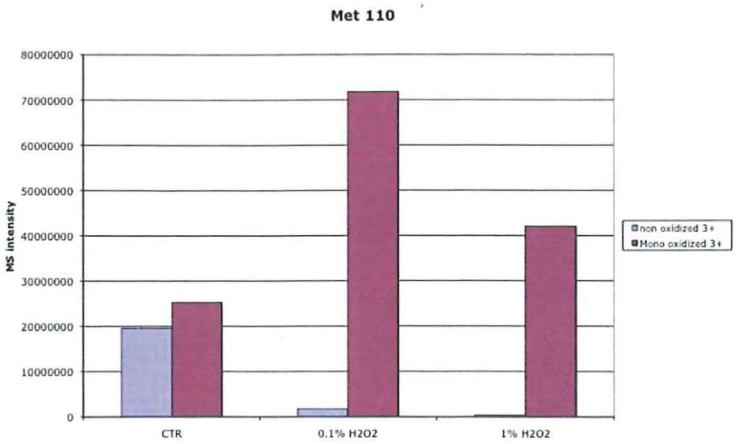
Categories among oxidation sites

	I	II	III	IV
alpha-chain	M110[1] M226 M259 M536			M495 H564
beta-chain	M220 M254 M335 M344 H400 M403 M456 M468	M397 M477		M148 C231 M272 W279
gamma-chain	M104 M120 M290	C35	C161 W279	

C=cysteine ; H=histidine ; M=methionine ; W=tryptophan

[1]Number according Swissprot (including peptide signal and fibrinopeptide)

I. Oxidized residues present in CTRL and appearing more oxidized after oxidative treatment (sensitive to oxidation)



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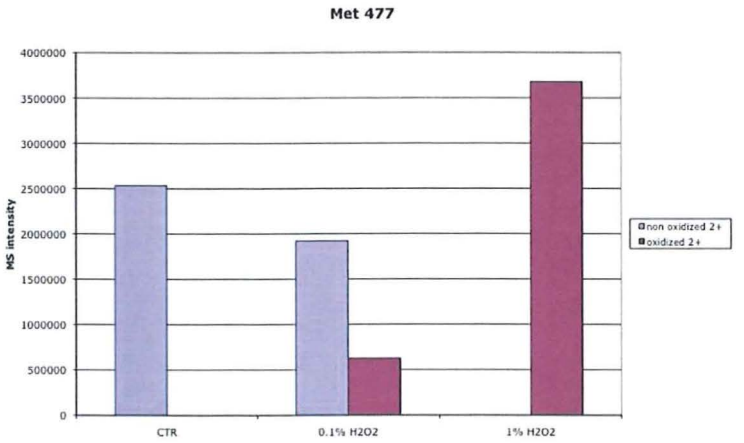
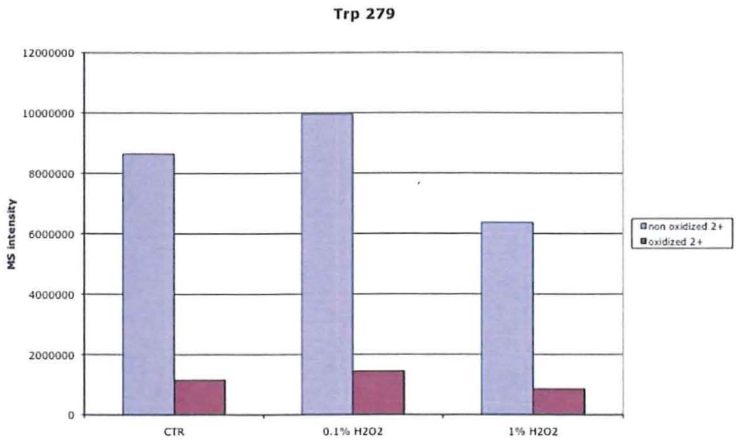
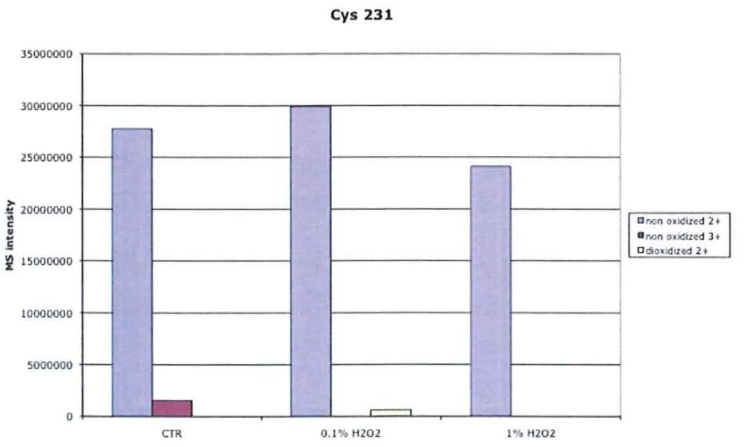


III. Residues appearing oxidized in CTRL and after oxidative treatment (insensitive to oxidation)



IV. Level of oxidation appearing in CTRL and/or after oxidative treatment (non-conclusive, because not enough signals are detected in MS)



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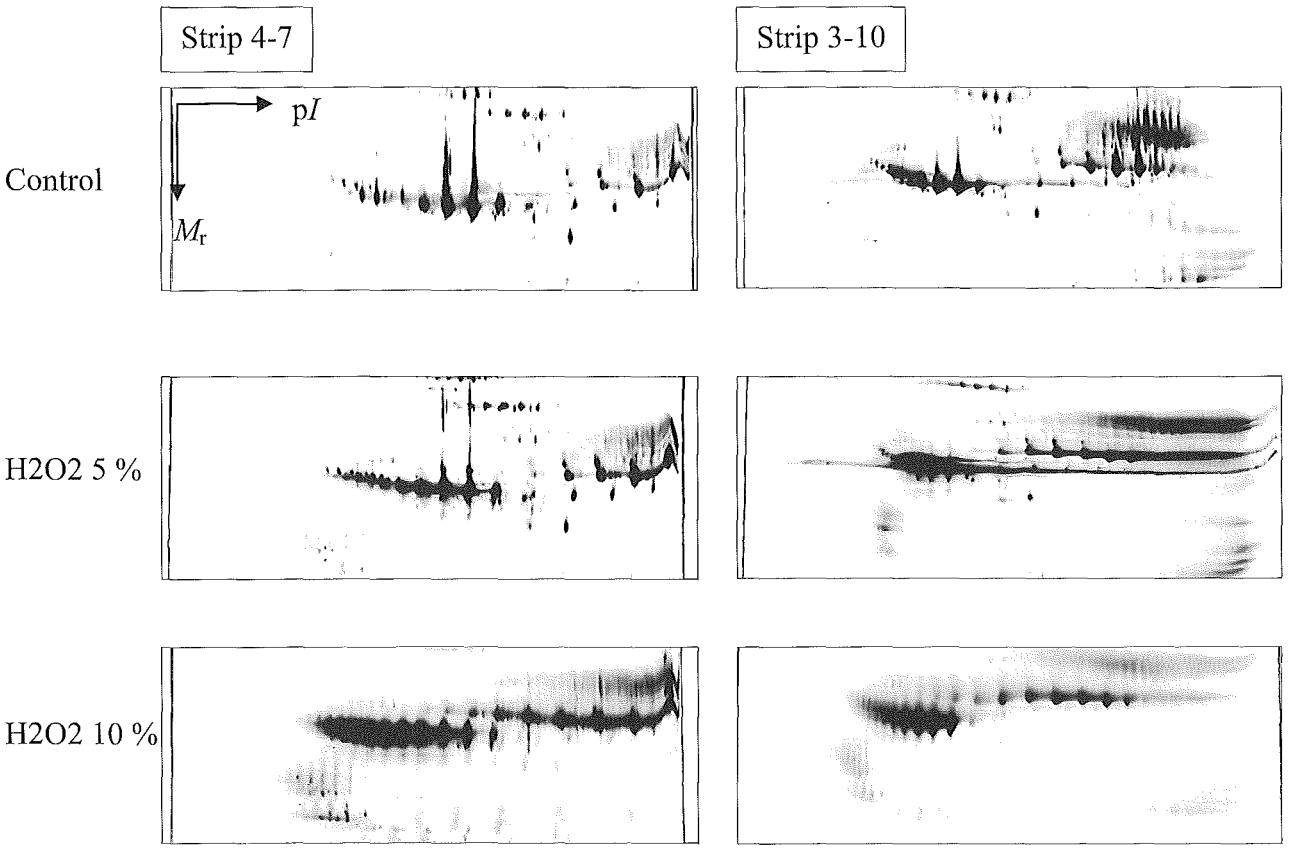

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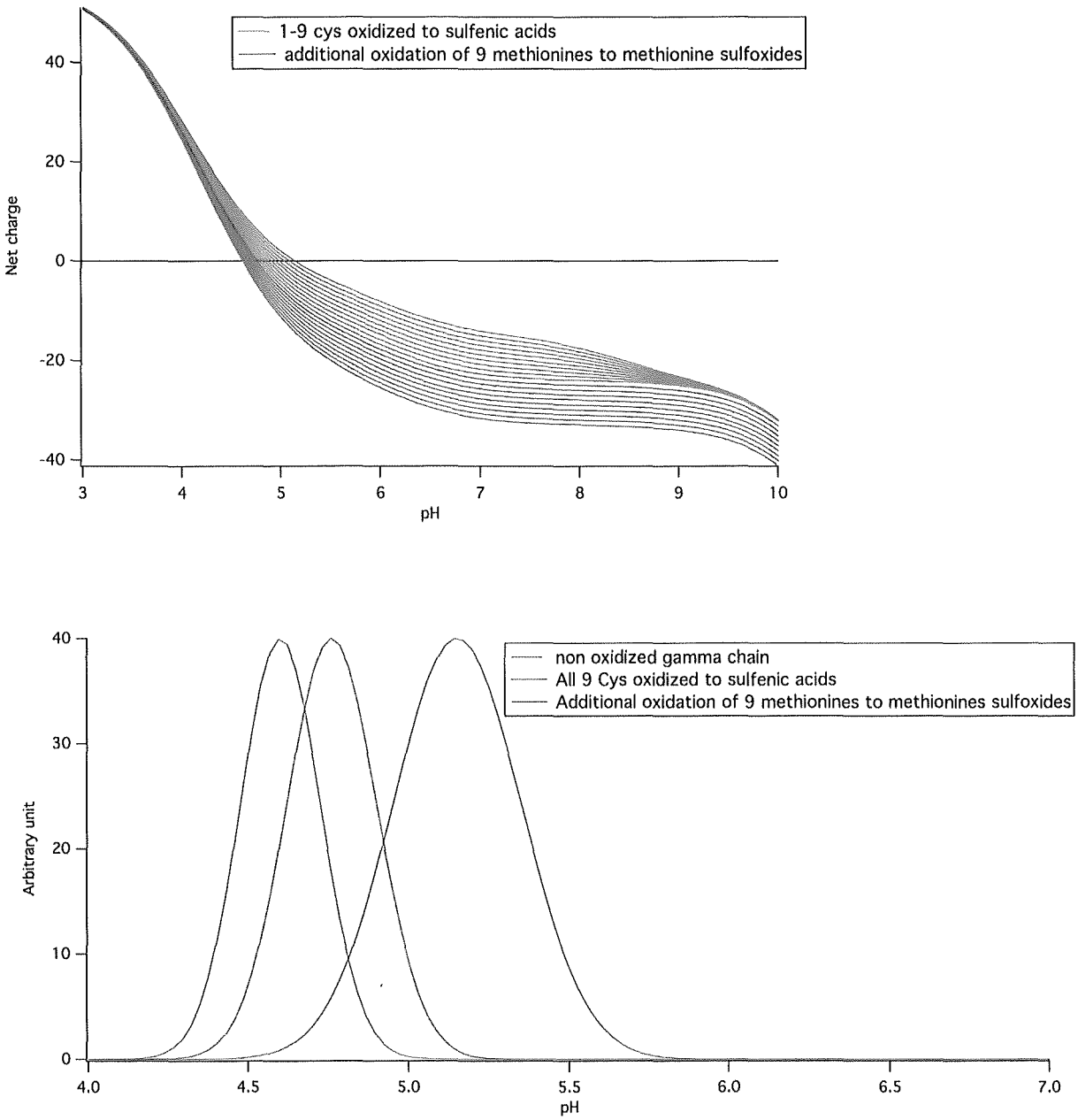

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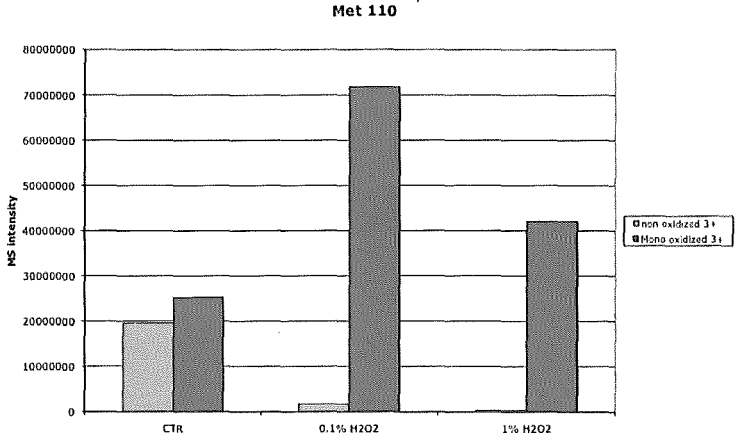

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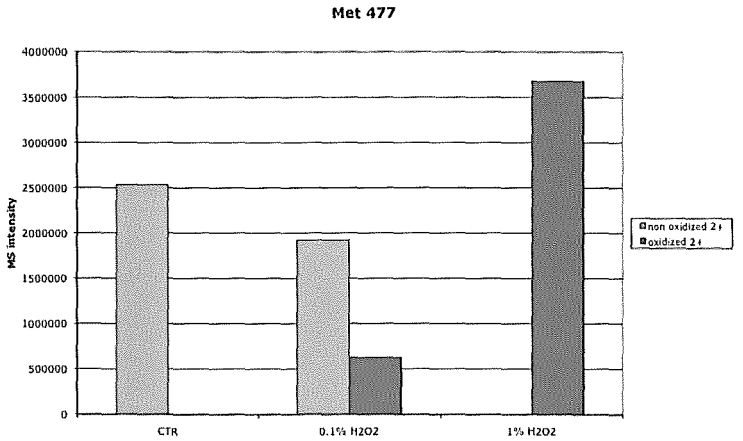

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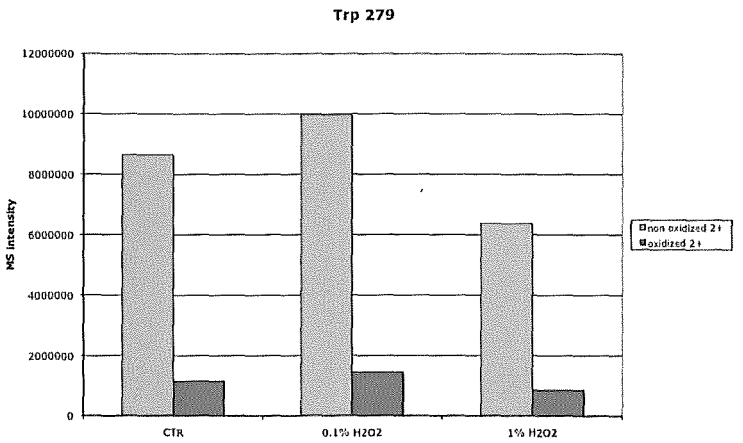

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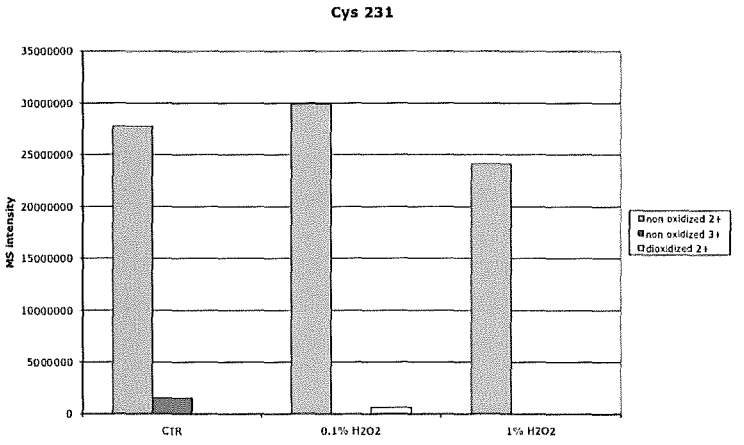

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