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Analysis of long-lived sulfur mustard-human hemoglobin adducts in blood samples by red blood cells lysis and on-line coupling of digestion on an immobilized-trypsin reactor with liquid chromatography-tandem mass spectrometry

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Abstract

As a highly alkylating chemical warfare agent, sulfur mustard reacts with blood proteins such as hemoglobin to form long-lived hydroxyethylthioethyl adducts that can be used as biomarkers of exposure. An optimized method was developed for the extraction of hemoglobin from blood samples. This procedure, involving the hemolysis of the red blood cells by freezing at -80°C in two cycles of 1 h, followed by the purification of the lysate by ultrafiltration on 100 and 50 kDa cutoff centrifugal devices, was then applied to the extraction of hemoglobin from blood samples spiked with sulfur mustard at different concentrations (ranging from 0.014 to 28 µg.mL^{-1}). More than 75% of the protein was extracted from the blood samples and the method demonstrated a satisfying repeatability, with a RSD of 12.6%. The extracted hemoglobin was then digested on-line on a laboratory-made trypsin IMER coupled with the analysis by liquid chromatography hyphenated with tandem mass spectrometry (LC-MS/MS) of the resulting alkylated peptides. A linear response was observed for the 13 alkylated peptides targeted for the sulfur mustard concentration range studied, with RSD down to 0.1% for the digestion repeatabilty. The limit of quantification of the method was estimated to be 0.4 ng.mL^{-1} as concentration of exposure to sulfur mustard in whole blood. Finally, a variation of the alkylation rates of hemoglobin was observed between the biological matrix and pure sample, since the preferential adduction sites in blood were the residues β-His^{97} and β-Val^{98}, both located on the alkylated peptide β-T11, while for purified hemoglobin in water, the residue β-His^{77} was the main adduction site. Thus, even though blood samples require an additional sample treatment step compared to pure standards, carrying out the study with whole blood allowed to collect information that are more representative of the phenomena occurring in the organism upon exposure to sulfur mustard.

Keywords

Human hemoglobin adducts; sulfur mustard; immobilized enzyme reactor; trypsin digestion; on-line coupling; LC-ESI-MS/MS.

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1. Introduction

Sulfur mustard (1,1'-thiobis(2-chloroethane)) is a severe cytotoxic, genotoxic and carcinogenic blistering chemical warfare agent, causing burns to the lungs, the eyes and the skin. Used for the first time during World War I [1], sulfur mustard (also known as HD) also caused multiple casualties during the Iran-Iraq military conflict [2,3]. Though this chemical agent is prohibited by the Organization for the Prohibition of Chemical Weapons (OPCW), there is still a need for fast, sensitive and accurate methods for the retrospective diagnosis of exposure to sulfur mustard. Indeed, due to its high toxicity, strong permeability, low volatility, ease of synthesis and lack of antidote, sulfur mustard represents a terrorist threat [4] and was used several times since 2015 in chemical terrorist attacks [5]. Moreover, accidental exposures to sulfur mustard were also reported in Europe and in China, due to the presence of old chemical weapons ammunitions in the environment [6–9].

As a strong vesicant and bi-functional alkylating agent, sulfur mustard can react with numerous nucleophiles present in the organism, such as proteins, to form hydroxyethylthioethyl (HETE) adducts, used as unequivocal biomarkers to retrospectively confirm an exposure to this chemical agent. The adducts that sulfur mustard forms with hemoglobin, the most abundant protein found in the red blood cells (RBCs) of human blood, are particular useful long-lived biomarkers. Indeed their life-span is similar to that of the native protein (*c.a.* 120 days) [10] and they can be detected up to several months after an exposure to sulfur mustard. Hemoglobin is a tetrameric protein composed of two chains of globin α and two chains of globin β. In the late 90s, eleven sites of alkylation were identified based on the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis of the tryptic digest of hemoglobin extracted from blood samples incubated with sulfur mustard, including the two N-terminal valine residues of both globin α and globin β chains that make up the protein (α-Val^{1} and β-Val^{1}), six histidine residues (α-His^{20}, α-His^{45}, α-His^{50}, β-His^{77}, β-His^{97} and β-His^{146}) and three glutamic acid residues (β-Glu^{22}, β-Glu^{26} and β-Glu^{43}) [11,12]. More recently, five new potential adduction sites were identified using a new analytical method developed in our laboratory (α-His^{72}, α-His^{87}, α-His^{89}, β-His^{2} and β-Val^{98}) on purified hemoglobin standard incubated with sulfur mustard in water [13]. The localization of the different adduction sites identified is presented on the **Fig.S1**. This method was developed as a complement to the modified Edman degradation procedure that is mainly used for the analysis of the adducts of sulfur mustard with the N-terminal valine residues of hemoglobin. In this time-consuming procedure, a cleavage agent, such as pentafluorophenyl isothiocyanate or phenyl isothiocyanate, is used to isolate the adducted N-terminal valine from the protein chain. This valine adduct is then analyzed by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS), after a potential derivatization step with heptafluorobutyrylimidazole or heptafluorobutyric anhydride [14,15]. The new analytical method developed in our previous study [13] was based on the LC-MS/MS analysis of the alkylated peptides obtained after the tryptic digestion of hemoglobin incubated *in vitro* with sulfur mustard in deionized water. This method was later improved by replacing the off-line in-solution digestion step by a digestion step on an immobilized enzyme reactor (IMER) [16], that presents several advantages compared to the conventional in-solution digestion. First of all, due to the immobilization of large amount of enzyme, the auto-digestion of the protease is greatly reduced and the high enzyme/substrate (E/S) ratio leads to shortened digestion times (32 times

shorter compared a digestion in solution). Moreover, the IMER can be reused numerous times with no cross contamination or loss of efficiency, thus reducing the long-term cost of the digestion and can be easily coupled on-line with the LC-MS/MS thus favoring the automation of the analysis of the resulting peptides [16]. In addition, it was demonstrated that the digestion on IMER was more repeatable than the in-solution digestion, with relative standard deviation (RSD) ranging between 1.0 and 20.9% for digestion on IMER and RSD varying between 5.7 to 53.3% for in-solution digestion [16].The developed IMER was applied to the digestion of standard hemoglobin in deionized water exposed *in vitro* to sulfur mustard concentrations ranging from 250 ng.mL^{-1} to 100 µg.mL^{-1}, followed by LC-MS/MS analysis. This method led to a satisfying linearity for all the 15 targeted alkylated peptides, and it was possible to detect less than two adducted chains over one million. However, this sensitivity was estimated in pure media.

Therefore, in this study, the IMER-LC-MS/MS method previously applied to hemoglobin standards was applied to the analysis of hemoglobin extracted from whole blood samples. A particular attention was paid on the optimization of the method used to extract hemoglobin from whole blood, particularly the two crucial steps of (i) red blood cells (RBCs) lysis procedure and (ii) the purification of the extracted hemoglobin by ultrafiltration. Then the whole optimized analytical procedure was applied to blood samples spiked with sulfur mustard at concentrations ranging from 0.014 µg.mL^{-1} to 28 µg.mL^{-1}.

2. Material and methods

2.1. Chemicals

Sodium chloride (NaCl), sodium hydrogen phosphate (Na_2HPO_4), sodium acetate (CH_3CO_2Na), sodium hydrogen carbonate ($NaHCO_3$), sodium azide (NaN_3), Trizma® hydrochloride ($NH_2C(CH_2OH)_3$.HCl, Tris-HCl) and calcium chloride dihydrate ($CaCl_2.2H_2O$) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France), as well as hydrochloric acid (HCl), formic acid (FA), trypsin from bovine pancreas treated with 6-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) and the cyanogen bromide-activated Sepharose 4B (CNBr-activated Sepharose) used for the trypsin immobilization. The BiCinchoninic acid Assay (BCA) protein assay reagents were obtained from Thermo Fisher Scientific (Courtaboeuf, France). Potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl) and acetonitrile HPLC grade (ACN) were provided by VWR (Fontenay-sous-bois, France) while high purity water was obtained using a Milli-Q water purification system (Millipore, Saint Quentin en Yvelines, France). The phosphate buffer saline solution (PBS, pH 7.4) consisted of 0.01 mol.L^{-1} Na_2HPO_4 and KH_2PO_4 and 0.15 mol.L^{-1} NaCl. The PBS-azide solution was a PBS solution containing 0.1% (w/w) of NaN_3. Amicon® Ultra-4 mL (100 kDa and 50 kDa) and Centrisart® (100 kDa) centrifugal ultrafiltration devices were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Regarding trypsin immobilization, the grafting solution and washing solutions were prepared as described in our previous study [16].

Non-spiked human blood sample (28 mL, batch 10200088369) from a healthy individual without known exposure to sulfur mustard was provided by the Centre de Transfusion Sanguine des Armées (CTSA, Clamart, France). Sodium citrate/citric acid was used as an anticoagulant. Another human blood sample (batch 1021002035) was obtained from the CTSA and was later spiked with sulfur mustard (DGA CBRN Defence, France, purity > 99% according to the NMR ^{1}D ^{1}H analyses) at concentrations of 0.014 µg.mL^{-1}, 0.07 µg.mL^{-1}, 0.14 µg.mL^{-1}, 0.7 µg.mL^{-1}, 1.4 µg.mL^{-1}, 5.6 µg.mL^{-1} and 28 µg.mL^{-1}. These spiked samples were prepared by the Analytical Chemistry department of DGA CBRN Defence center by adding 20 µL of sulfur mustard at 1.4 µg.mL^{-1}, 7 µg.mL^{-1}, 14 µg.mL^{-}

[1], 70 µg.mL^{-1}, 0.14 mg.mL^{-1}, 0.56 mg.mL^{-1} or 2.8 mg.mL^{-1} in isopropanol (iPrOH), respectively, to 2 mL of blood. The mixtures were then incubated overnight (*c.a.* 19 h) at room temperature under horizontal stirring and were then stored at 4°C until further use. In parallel, two samples were used as negative controls using the same batch (1021002035) (i) 2 mL of unmodified whole blood and (ii) 2 mL of blood to which 20 µL of iPrOH were added. Both control samples were stirred at room temperature overnight.

2.2. Apparatus and LC-MS/MS analysis

The experimental set-up used for the on-line enzymatic digestion of hemoglobin on the trypsin IMER and the analysis of the resulting peptides by LC-MS/MS, is described in **Fig.S2**. It was composed of three six-port switching valves. The first valve was connected to the injection loop (20 µL) and to a syringe pump (KDS 100 Infusion Pump, KD Scientific). The second valve was connected to the trypsin IMER precolumn, which was set in an oven (Crococil oven, CIL) at 25°C. The last valve was connected to a desalting column that consisted of a polymeric reversed phase trapping precolumn (Newguard RP-18, 15 mm x 3.2 mm i.d., 7 µm, Perkin Elmer, Villebon-sur-Yvette, France), to the analytical column (Atlantis® dC$_{18}$, 150 mm x 2.1 mm i.d., 3 µm, 100 Å, Waters, Guyancourt, France) and to the binary analytical pump (Dionex Ultimate 3000 pump, Thermo Scientific, controlled by Chromeleon 6.8 SR11). Detection of the peptides was performed with a triple quadrupole mass spectrometer (TSQ Quantum Access Max®, Thermo Scientific) equipped with an electrospray (ESI) ion source (IonMax source, probe HESI-II, Thermo Scientific). The mobile phase was composed of a mixture of deionized water (A) and acetonitrile (B) acidified with 0.1% of formic acid, with a flow rate of 200 µL.min^{-1}. A gradient composed of 100% A during 5 min followed by an increase from 0% to 40% B in 80 min prior to a plateau at 40% B during 2 min was used for the peptides separation. The peptides were then detected by mass spectrometry in multiple reaction monitoring (MRM) mode. Ionization was performed in positive mode with a source voltage of 3,000 V, capillary and vaporizer temperatures were respectively set at 230°C and 277°C, the sheath gas pressure was set at 30 psi and the skimmer offset was set at 1V. A scan width of 0.5 m/z and a dwell time of 0.35 s were used for the analysis. The mass parameters and the MRM transitions used for each peptide were previously optimized [16] and are detailed in the **Table S1**. The limits of detection (LOD) and limits of quantification (LOQ) were estimated by calculation for a Signal/Noise (S/N) ratio of 3 and 10, respectively.

2.3. Synthesis of the trypsin-based IMER

The trypsin-based IMER was prepared following the protocol described in a previous study [16]. Briefly, CNBr-Sepharose was swollen in an Eppendorf lobind with a HCl solution (10^{-3} mol.L^{-1}) and was then washed with a sodium carbonate buffer ($NaHCO_3$ 0.1 mol.L^{-1}, NaCl 0.5 mol.L^{-1}, pH 8.3). After elimination of the supernatant, a trypsin solution prepared in the coupling sodium carbonate buffer were added to the Sepharose and the mixture was incubated at 4°C during 16 h before being packed into a 20 x 2.1 mm i.d. precolumn. The remaining uncoupled sites were then blocked using a Tris buffer (Tris-HCl 0.1 mol.L^{-1}, pH 8.0) and the non-bounded enzyme was further removed, by alternatively washing the grafted gel with a low-pH buffer solution (0.1 mol.L^{-1} acetate buffer, NaCl 0.4 mol.L^{-1}, pH 4.0) and a high-pH buffer solution (0.1 mol.L^{-1} carbonate buffer, NaCl 0.5 mol.L^{-1}, pH 8.3). The IMER was then stored during several months, when not used, at 4°C in a PBS solution containing 0.1% azide.

As previously described, the amount of trypsin grafted on Sepharose was evaluated by a BiCinchoninic acid (BCA) assay applied to the determination of non-grafted trypsin in the supernatant and in the washing fractions [16].

Trypsin grafting yield was determined by dividing the amount of trypsin remaining in the supernatant and the washing solutions after the immobilization step by the initial amount of trypsin in the coupling solution.

2.4. *Development of an extraction and purification procedure of hemoglobin from whole blood samples*

The development and optimization of the extraction of hemoglobin from whole blood procedure was performed using non-spiked human blood samples. Several parameters, including the RBCs hemolysis method, number of cycles of hemolysis and the type of 100 kDa cutoff filters used for the purification of the lysate, were studied and compared.

2.4.1. *Hemolysis methods*

Two methods were applied to the hemolysis of the RBCs, in order to determine which method allowed to extract the greatest amount of hemoglobin. To do so, 4 mL of fresh blood were centrifuged at 4,500 rpm (centrifuge Heraeus Labofuge 400R, Thermo Scientific, Courtaboeuf, France) during 10 min at 4°C (protocol inspired by Noort *et al*. [11]). After elimination of the plasma and the blood platelets, the RBCs were washed three times with an equivalent volume of saline solution (0.9% NaCl, w/v) followed by centrifugation at 2,000 rpm during 10 min and at 4°C, in order to remove the residual plasmatic proteins. Two equivalent volumes (*c.a*. 4 mL) of deionized water were then added to the RBCs and the diluted RBCs fraction was then separated into two equal fractions, each of them submitted to a different hemolysis method. One fraction was frozen at -80°C during 1 h (lysis method currently used at DGA CBRN Defense center and similar to that used by Zhang *et al*. [17]), while the other one was lysed using a sonication probe (Vibra-Cell®, VWR, Fontenay-sous-Bois, France) on ice at 130 W during 1 min, repeated five times, with a 1 min break between two sonication steps to prevent the sample heating and the hemoglobin degradation [18]. An illustrative summary explaining the experimental strategy is presented in **Fig.S3A**.

The lysates resulting from the two hemolysis methods were centrifuged at 4,500 rpm during 10 min at 4°C in order to eliminate the cell debris and the non-lysed RBCs by sedimentation. Then, the supernatants were transferred to a 100 kDa cutoff Amicon® centrifugal device and were filtered by ultrafiltration at 4,500 rpm during 10 min and at 4°C. The filtrates containing the hemoglobin (MW=64,458 Da) were then filtered by ultrafiltration on a 50 kDa cutoff Amicon® centrifugal device. The hemoglobin trapped on the filters was then recovered and quantified using a BCA assay (see section 2.4.4). The procedure used to filter and purify the lysates is illustrated on **Fig.S3B** and described in section 2.4.2. Each lysis procedure was independently repeated three times (n=3).

2.4.2. *Purification of hemoglobin with 100 kDa cutoff filters*

After selection of the best hemolysis procedure, two types of 100 kDa cutoff filters were studied in order to select the most efficient for the purification of hemoglobin extracted from whole blood: the Ultra-4 mL Amicon® and the Centrisart® centrifugal devices. To study their efficiency, the lysate (resulting from the freezing at -80°C during 1 h of the RBCs isolated from 4 mL of whole blood and washed according to the procedure described in section 2.4.1.) was divided into two equal fractions and each fraction was centrifuged at 6,400 rpm during 3 min using a HF-120 Tomy capsule (Tomy Tech, Palo Alto, USA). The supernatants were then filtered in the same conditions (4,500 rpm during 10 min at 4°C), either on an Amicon® filter or on Centrisart® filter, both of them having a 100 kDa cutoff (see **Fig.S4A**). The filtrates were then collected and filtered again by ultracentrifugation at 4,500 rpm during 10 min and at 4°C on a 50 kDa cutoff Amicon® centrifugal filter (see **Fig.S4B**). The

hemoglobin trapped on the filters was then recovered and quantified using a BCA assay (see section 2.4.4). Each filtration method was independently repeated three times (n=3), in order to compare the quantity of hemoglobin recovered with both 100 kDa cutoff devices.

2.4.3. *Hemolysis number of cycles*

In order to measure the influence of the number of freezing/thaw cycles on the quantity of hemoglobin recovered, 2 mL of whole blood were treated as previously (description in section 2.4.1 and 2.4.2) except that the diluted RBCs were separated into two equal fractions of 1.5 mL and a volume of 1 mL of deionized water (*i.e.* two equivalent volumes with respect to the RBCs initially present in the whole blood sample) was added to the first fraction, which was frozen at -80°C during 2 h (1 cycle). The second fraction was frozen at -80°C during 1 h, as mentioned in the previous sections, and, after thaw at room temperature, the resulting lysate was centrifuged at 6,400 rpm during 5 min. The supernatant was collected and transferred to a 100 kDa cutoff Amicon® filter, while 1 mL of deionized water was added to the non-lysed RBCs that had sedimented after the centrifugation at 6,400 rpm and the mixture was one more time frozen at -80°C during 1 h (2 cycles of 1 hour), (see **Fig.1A**).

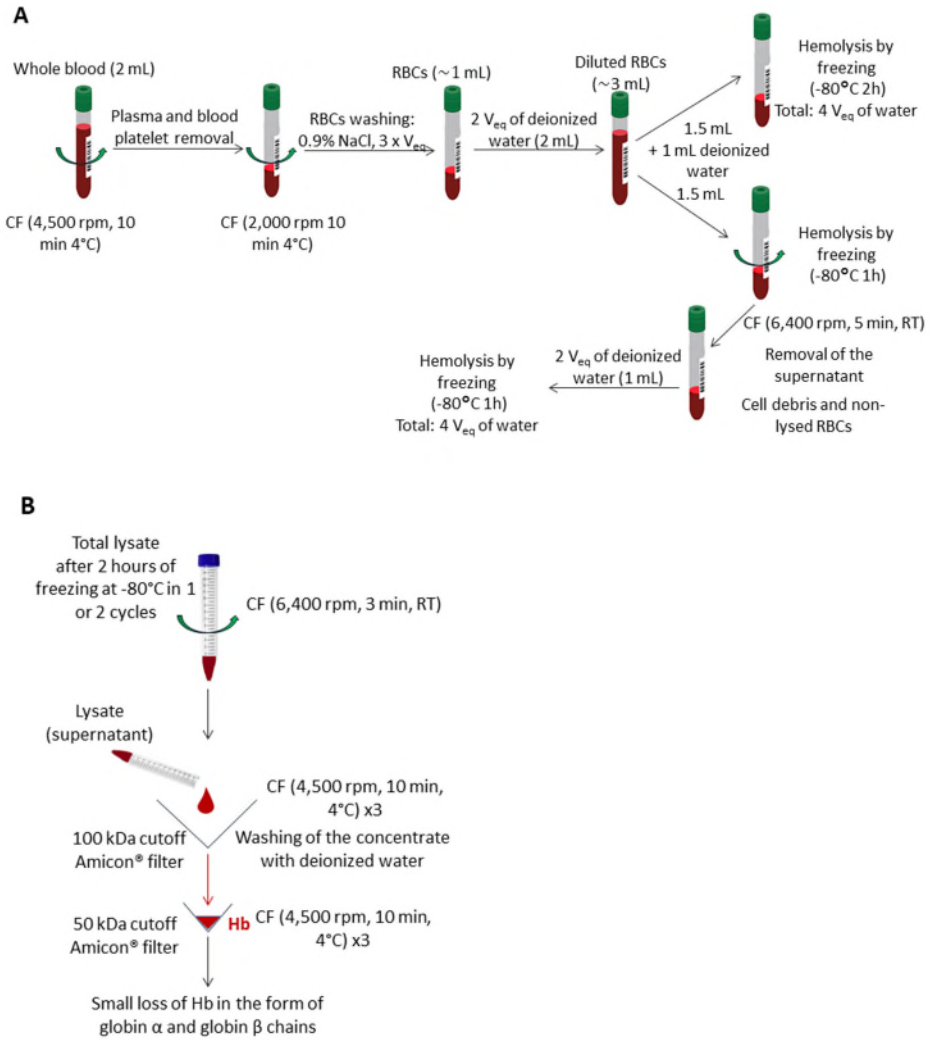

Fig.1. Schematic representation of the isolation of RBCs from whole blood and their hemolysis by freezing at -80°C during 2 hours in one or two cycles (**A**) followed by the lysate filtration and purification of the extracted hemoglobin by ultrafiltration on 100 and 50 kDa cutoff Amicon® filters (**B**). The steps retained for the treatment

of blood samples spiked with sulfur mustard are framed. V_{eq}: equivalent volume, RT: room temperature. See assay 3 in Table 1 for experimental values of extracted Hb obtained with both hemolysis methods. CF: centrifugation.

After thaw at room temperature, both lysates were centrifuged at 6,400 rpm during 5 min and the supernatants were transferred to 100 kDa cutoff Amicon® centrifugal devices, while the cell debris and the non-lysed RBCs were washed with 0.5 mL of deionized water before being centrifuged at 6,400 rpm at room temperature during 5 min. This washing step was repeated until the supernatant was lightly colored. For the hemolysis performed in one or two cycles, the supernatants recovered after each washing step were gathered and transferred into the 100 kDa cutoff Amicon® filter. After ultrafiltration as described in the section 2.4.2, the filtrates containing the hemoglobin were transferred into the 50 kDa cutoff Amicon® filter and were filtered as previously mentioned (see **Fig.1B**). The hemoglobin trapped on the filters was then recovered and quantified using a BCA assay (see section 2.4.4). The hemolysis by freezing at -80°C during 2 h, in one or two cycles, were repeated twice each, in order to evaluate the influence of the number of freezing/thaw cycles on the quantity of hemoglobin extracted from blood samples.

2.4.4. Quantification of hemoglobin extracted from whole blood samples

After dilution by a factor 200, a BiCinchoninic acid (BCA) assay was used to quantify the hemoglobin extracted from non-spiked blood samples and from blood samples exposed *in vitro* to sulfur mustard at concentration levels ranging from 0.014 to 28 µg.mL^{-1}. A linear calibration curve was made using solutions of pure hemoglobin in the Tris-HCl (pH 8.0) buffer at concentrations of 100 µg.mL^{-1}, 250 µg.mL^{-1}, 500 µg.mL^{-1}, 750 µg.mL^{-1}, 1 mg.mL^{-1}, 2 mg.mL^{-1} and 4 mg.mL^{-1} and by adding 10 µL of each solution in triplicate to the pits of a 96-well microplate. After addition of the working reagent to each microwell, the microplate was incubated at 37°C during 30 min, with agitation at 300 rpm using a PHMP Grant-Bio thermoshaker (Dutscher, Bernolsheim, France). The absorbance in the pits was then measured at 562 nm with a spectrophotometer (SpectraMax M2, Molecular Devices, St Gregoire, France). The amount of hemoglobin lost under the form of free chains of globin α (MW=15,126 Da) and globin β (MW=15,867 Da) was also estimated using a BCA assay. The calculation of the percentage of hemoglobin extracted from blood samples was performed considering an average concentration of hemoglobin in human blood of 150 mg.mL^{-1} (classical range: 120-180 mg.mL^{-1}).

Hemoglobin extracts were diluted in the Tris-HCl buffer (pH 8.0) to obtain a final concentration of hemoglobin of 1 mg.mL^{-1} for each concentration of sulfur mustard studied, prior to the digestion of the extracted alkylated hemoglobin on the trypsin IMER.

2.5. On-line digestion of hemoglobin on IMER

The protocol used for the digestion on the trypsin IMER was described in our previous study [16]. Briefly, 20 µg of hemoglobin extracted from blood sample spiked with sulfur mustard were loaded into a 20 µL injection loop and were then transferred to the trypsin IMER with the Tris-HCl buffer (pH 8.0). After digestion of the protein by *stop flow* at 25°C during 30 min, the resulting peptides were transferred to the desalting precolumn and the salts were removed by percolating deionized water (see **Fig.S2**). The peptides were then transferred from the trap column to the analytical column with the mobile phase by switching the third valve and were analyzed by LC-MS/MS. The trypsin IMER can be directly reused after a simple washing step with the Tris-HCl buffer during 30 min between two digestions. In this study, the IMER was used 27 times and no loss of efficiency nor cross contamination were observed.

2.6. Statistical test

The Student test with a confidence level of 95% (α=0.05) mentioned in this paper was performed with the online software Statistical Tools for High-Throughput Data Analysis (STHDA).

3. Results and discussion

3.1. Development of an extraction and purification procedure of hemoglobin from whole blood samples

3.1.1. Comparison of two hemolysis methods

The RBCs present in 4 mL of human whole blood were separated from the plasma and blood and were then washed with a saline solution (see **Fig.S3A** and section 2.4.1). Two equivalent volumes of pure water were added to the washed RBCs, than were then separated in two equal fractions and each fraction was submitted to a different hemolysis method: a conventional hemolysis procedure by freezing [17] and a hemolysis performed by sonication, based on a study by Andrade *et al.*[18] who compared several hemolysis methods. The hemolysis by freezing was carried out by putting the first fraction into a freezer at -80°C during 1 h, while the sonication procedure was carried out on ice with the second fraction at 130 W during one minute, repeated five times, to prevent the degradation of the sample. The lysates resulting from both hemolysis methods were then centrifuged at 4,500 rpm during 10 min at 4°C, before being filtered and purified according to the procedure described in **Fig.S3B** and detailed in the section 2.4.1., using 100 and 50 kDa Amicon® cutoff filters. Indeed, since the molecular weight of human hemoglobin is 64,458 Da, the protein passes through the 100 kDa cutoff filter while the big proteins and cell debris present in the RBCs lysate are trapped on the filter. Then, the filtrate containing the hemoglobin is transferred to the 50 kDa cutoff filter, thus allowing to remove the small proteins and salts while hemoglobin is trapped on the membrane and therefore purified. The results obtained during the comparison of the two hemolysis methods are reported in the **Table 1** (assay 1).

Regarding the hemolysis of the RBCs by sonication, a good separation of the phases was observed after centrifugation of the lysate at 4,500 rpm during 10 min at 4°C. Indeed, a red precipitate, corresponding to the non-lysed RBCs and cell debris, was obtained at the bottom of the centrifuge tubes and could easily be eliminated. However, regarding the hemolysis carried out by freezing at -80°C during 1 h, only one phase was obtained after the centrifugation step, thus meaning that the cell debris were smaller than those resulting from the sonication procedure and that more RBCs were lysed. Consequently, the whole lysate was transferred to the 100 kDa cutoff Amicon® filter, while, in the case of the hemolysis performed by sonication, only the supernatant, *i.e.* the lysate, was transferred to the filter after removal of the cell debris and non-lysed RBCs.

Important difficulties were encountered for the filtration on the 100 kDa cutoff Amicon® filter of the whole lysate resulting from the freezing procedure, even after its dilution by addition of 1 mL of deionized water. Indeed, the cell debris present in the lysate have clogged the filter membrane, which made the filtration very laborious and led to an important loss of hemoglobin. On the contrary, the filtration of the lysate obtained after hemolysis by sonication was faster, more efficient and less laborious.

During the second filtration step on the 50 kDa cutoff Amicon® centrifugal devices, a fast and efficient filtration was obtained for both fractions resulting from the two hemolysis methods. As expected, based on the deep red color of the concentrate, it was observed that hemoglobin was trapped on the filter, thus meaning that the protein was under its tetrameric form (MW=64,458 Da) after the hemolysis step, independently of the hemolysis

procedure. A slight orange coloration of the filtrate was observed for both hemolysis methods, implying than a small fraction of the hemoglobin was under the form of free globin α and globin β chains. Based on the average concentration of hemoglobin in human blood, the estimation of the hemoglobin loss performed with a BCA assay revealed than less than 1% of the protein was under the form of free chains in the filtrate.

The amounts of hemoglobin extracted from whole blood, and trapped on the 50 kDa cutoff Amicon® filter, obtained with both hemolysis methods were estimated using a BCA assay and the results are presented in the first two columns of the **Table 1**. Since hemoglobin represents more than 98% of the cytoplasmic content of RBCs, the concentrations evaluated with the BCA assay performed after the filtration steps can be considered as only purified hemoglobin. The hemolysis performed by freezing at -80°C allows to extract more hemoglobin from the same blood sample, with an average amount of 98.8 mg (RSD=2.7%, n=3) of hemoglobin, than the hemolysis carried out by sonication, for which the mean quantity of hemoglobin extracted was only 68.0 mg (RSD=45.2%, n=3). Therefore, knowing that human blood contains on average 150 mg of hemoglobin per mL, the theoretical quantity of hemoglobin present in 2 mL of whole blood would be 300 mg. Even though this is an average value and hemoglobin concentrations car vary from an individual to another, this value is a good way to estimate the potential of a method, especially when comparing two procedures with the same blood sample. Based on this estimation, the percentage of hemoglobin recovered after hemolysis by sonication is 22.7%, while that recovered after the hemolysis by freezing is 32.9%. Moreover, after three repetitions of the hemolysis procedures in the same conditions, it appeared that the hemolysis by freezing was more repeatable than the sonication method, since the relative standard deviations (RSD) for the quantity of hemoglobin extracted were 2.7% and 45.2%, respectively. This result could be explained by the fact that the hemolysis performed by sonication led to the formation of a brown foam that stayed above the lysate after the centrifugation, thus forming a solid deposit impossible to filtrate. Therefore, it was necessary to remove this foam in order to be able to collect the lysate for the ulterior filtration step, but this removal was rather laborious and involved one more time the experimentalist, which can lead to a loss of repeatability. The hemolysis by sonication was therefore abandoned in favor of the freezing approach. Nevertheless, even if the hemolysis by freezing allows to extract larger amounts of hemoglobin than the hemolysis by sonication, it is important to note that only 32.9% of the hemoglobin initially present in the whole blood sample could be recovered following the two filtration steps. Indeed, an important loss of hemoglobin could be observed on the concentrate of the 100 kDa cutoff Amicon® filter for the lysate resulting from the freezing of the RBCs. Therefore, an optimization of this filtration step was necessary in order to reduce the loss of hemoglobin.

3.1.2. Comparison of two 100 kDa cutoff filters for hemoglobin purification

Since the hemolysis by freezing at -80°C during 1 h appeared as the most efficient and most repeatable procedure to lyse the RBCs, this method was applied to the RBCs isolated from 4 mL of whole blood (referred as assay 2) and washed with a saline solution (see **Fig.S4A**). After thaw at room temperature, the resulting lysate was then divided into two fractions and each fraction was centrifuged at 6,400 rpm at room temperature during 3 min (instead of 4,500 rpm during 10 min at 4°C). This increase of the centrifugation speed allowed the elimination of the cell debris. Indeed, unlike the previous tests, a sedimentation of the non-lysed RBCs and the cell debris as a brown precipitate was obtained after this centrifugation step at 6,400 rpm. The supernatants were then collected and transferred either to a 100 kDa cutoff Amicon® filter or to a 100 kDa cutoff Centrisart® filter (see **Fig.S4B**). For both types of centrifugal devices, the centrifugation was carried out similarly (see section 2.4.2). The

Centrisart® filter present a potential interest since the filtration occurs in the opposite direction of the centrifugal force, which could prevent the clogging of the membrane previously observed with the Amicon® filter and, therefore, could lead to a faster filtration of the lysates while reducing the loss of hemoglobin.

In the case of the Amicon® filter, the filtration turned out to be fast and simple and, after three 10 min cycles of filtration with the addition of 1 mL of deionized water each time, only a small loss of hemoglobin was observed on the membrane. Therefore, the removal of the cell debris by increasing the centrifugation speed has considerably simplified the filtration step on the 100 kDa cutoff Amicon® device. On the contrary, the lysate filtration on the 100 kDa cutoff Centrisart® device turned out to be more laborious. First, the maximal volume that can be filtered is 2.5 mL, while up to 4 mL can be filtered with the Amicon® filter, which requires to perform the filtration in several steps and thus requires more time. Moreover, even after more than 1 h of filtration (cumulated duration), it has been observed, based on the deep-red color of the concentrate that hemoglobin could not go through the membrane and remained mainly in the concentrate. Consequently, only a very small proportion of the protein present in the lysate was recovered during the filtration step on the 100 kDa cutoff Centrisart® filter.

The filtrates containing the hemoglobin were then filtered on a 50 kDa cutoff Amicon® centrifugal device. The filtration, performed at 4,500 rpm and at 4°C, was fast and simple whether the first filtration step had been carried out on a 100 kDa Amicon® or Centrisart device. Indeed, a total filtration was achieved in 30 min, *i.e.* three cycles of 10 min centrifugation. In addition, similarly to what was observed during the comparison of the hemolysis methods in section 3.1.1, a light orange coloration of the filtrates was noted after the filtration on the 50 kDa cutoff filter, thus meaning that a part of the hemoglobin was under the form of free globin α and globin β chains. Nevertheless, a BCA assay highlighted the fact that this loss of hemoglobin as free chains only represented a small fraction, respectively less than 2% and 1% of the protein extracted from whole blood after filtration on 100 kDa cutoff Amicon® or Centrisart® filters.

The hemoglobin trapped on the 50 kDa cutoff Amicon® filters was collected before being quantified using a BCA assay. As seen in the results presented in the second column of the **Table 1** (assay 2), and confirming the visual observations previously described, an important part of hemoglobin remains on the membrane during the lysate filtration step on the 100 kDa cutoff Centrisart® filter. Only 6.3% of the hemoglobin theoretical quantity was extracted from whole blood. This result was obtained with a good repeatability for the three replicates carried out, with an average hemoglobin quantity of 18.9 mg and a RSD of 6.2%. In comparison, the filtration with the 100 kDa cutoff Amicon® filter allowed the extraction of more hemoglobin, with an average quantity of 95.6 mg, corresponding to 31.9% of the theoretical hemoglobin amount present in the blood sample. Moreover, a satisfying repeatability was observed for the three replicates, with a RSD of 5.7%. Therefore, the 100 kDa cutoff Amicon® filters were kept for the lysates purification procedure. It must also be noted that the results obtained with the 100 kDa cutoff Amicon® filters (assay 2, right column) are similar to those obtained during the comparison of the RBCs hemolysis methods, for a hemolysis by freezing at -80°C during 1 h (assay 1, left column). Thus, it could be concluded that the increase of the centrifugation speed after the hemolysis step eases the filtrations steps without affecting the extraction yields. Nevertheless, since the hemoglobin extraction percentage is relatively low, an optimization of the number of freezing/thaw cycles was carried out, in order to study its influence on the quantity of hemoglobin extracted from whole blood.

3.1.3. Influence of the number of hemolysis cycles on the recovered amount of hemoglobin

In order to measure the influence of the number of freezing/thaw cycles on the extracted amount of hemoglobin, 2 mL of whole blood were centrifuged and the isolated RBCs were washed before their dilution with deionized water and their separation in two fractions. The fractions were then frozen at -80°C during 2 h or two cycles of 1 h (see **Fig.1A**). It must be mentioned that because of the lack of biological samples, only 2 mL of blood were used for these experiments, and not 4 mL like in the experiments of previous assays. Moreover, it is important to note that, after the blood centrifugation step, the plasma obtained was slightly red-colored, thus meaning that the RBCs were already partially lysed and that a fraction of the hemoglobin was lost in the plasma due to the degradation of the blood sample.

After thaw at room temperature, the lysates were centrifuged at 6,400 rpm (5 min) and the supernatants were transferred to a 100 kDa cutoff Amicon® centrifugal device (see **Fig.1B**), while the precipitates were washed several times with deionized water until the supernatant was slightly colored (see **Fig.S5**), to recover a maximum amount of hemoglobin. The supernatants collected after each washing step were gathered and filtered on the 100 kDa Amicon® filter at 4,500 rpm. Like previously mentioned in the section 3.1.2., the lysates filtration was fast and simple and was complete after three cycles of 10 min (see **Fig.1B**).

The resulting filtrates were then transferred to the 50 kDa cutoff Amicon® centrifugal devices. The losses of hemoglobin as free chains of globin α and globin β in the filtrates were estimated with a BCA assay to be inferior to 3% of the initial quantity of hemoglobin present in the blood samples, for the hemolysis performed in one or two cycles.

As reported in **Table 1** (assay 3), amounts of 94.9 to 101.7 mg of hemoglobin were obtained after the hemolysis performed in two cycles, while 80.6 to 87.0 mg were recovered for the hemolysis in one cycle (see the last column of **Table 1**). These quantities correspond respectively to the extraction of 63.3-67.8% and 53.7-58.0% of the hemoglobin theoretically present in the initial blood samples. Therefore, it appeared that the hemolysis of the RBCs by freezing at -80°C in two cycles of 1 h with two equivalent volumes of deionized water was more efficient than the hemolysis carried in one cycle of 2 h with four equivalent volumes of water and led to the recovery of more important quantity of hemoglobin. The hemolysis in two cycles was thus selected for the rest of this study since it also seemed to be more repeatable, even if only two replicates were carried out due to the lack of biological material. However, the experiments performed on blood samples exposed to sulfur mustard and described in the following section confirmed the results obtained during the development of the extraction procedure with non-spiked blood samples.

3.2. Application of the optimized procedure to blood samples exposed to sulfur mustard

The procedure that includes two 1 h cycles of freezing at -80°C (see **Fig.1**) was applied to the extraction of hemoglobin from a new batch of blood sample (1 mL) *in vitro* exposed to different concentration levels of sulfur mustard: 0.014 µg.mL^{-1}, 0.07 µg.mL^{-1}, 0.14 µg.mL^{-1}, 0.7 µg.mL^{-1}, 1.4 µg.mL^{-1}, 5.6 µg.mL^{-1} and 28 µg.mL^{-1}. For each of these concentrations, the final percentage of iPrOH, solvent used in the spiking solution, in whole blood was set at 1%. As negative controls, the procedure was also applied to the extraction of hemoglobin present in a non-spiked blood sample with or without the addition of 1% of iPrOH, in order to evaluate the potential influence of the solvent on the extraction of the protein.

The results obtained after the quantification of the hemoglobin extracted from these nine blood samples mentioned above are reported in the **Table 2**. Based on these results, it appeared that the addition of a small percentage of

iPrOH in the blood samples did not have any impact on the quantity of hemoglobin extracted, since the result obtained for the non-spiked blood samples containing 1% of iPrOH was similar to that obtained for the non-spiked sample of reference (127.7 and 124.4 mg of hemoglobin extracted, respectively). Moreover, the results have also highlighted the fact that the presence of sulfur mustard in the samples had no influence on the extraction of the native hemoglobin and the hemoglobin-HETE adducts. Indeed, a satisfying repeatability was obtained for all the samples treated, with a RSD of 12.6% (n=9) for a mean quantity of hemoglobin extracted of 114.3 mg. However, it was also noted that, in the case of the blood samples spiked with sulfur mustard at concentrations of 0.07 $\mu g.mL^{-1}$ and 1.4 $\mu g.mL^{-1}$, the quantities of hemoglobin extracted (82.3 and 96.7 mg, respectively) were inferior to those obtained for the other sulfur mustard concentrations. Since all the samples were treated under strictly similar conditions, one hypothesis that could explain this result would be that these two samples contained less hemoglobin than the other samples prior to the hemolysis. Indeed, as illustrated on the **Fig.S6**, after addition of two equivalent volumes of deionized water to the washed RBCs before the first hemolysis cycle, it was observed that red color of the samples spiked with sulfur mustard at 0.07 $\mu g.mL^{-1}$ and 1.4 $\mu g.mL^{-1}$ was less intense than that of the other samples (as illustrated by sample exposed to sulfur mustard at 0.14 $\mu g.mL^{-1}$ taken as model sample (**Fig.S6C**)), thus implying that the hemoglobin concentration was lower. A more important loss of hemoglobin in the plasma during the first centrifugation of the blood samples could explain this difference, knowing that all the samples were obtained from the same blood donor. Nevertheless, considering the numerous sample treatment steps applied on the blood samples, a RSD of 12.6% is very acceptable.

The mean quantity of hemoglobin extracted from the spiked blood samples (114.3 mg) corresponded to 75.6 % of the protein theoretically present in the samples. This percentage is superior to that obtained during the development of the extraction procedure with non-spiked blood samples (range 63.3-67.8%). This difference might be explained by the fact that, during the optimization of the method, the blood samples were aged and the RBCs had undergo a hemolysis prior to their treatment, thus leading to a loss of hemoglobin in the plasma and to an under-estimation of the extraction percentage. Nevertheless, the results obtained for the spiked blood samples were satisfying, both in terms of repeatability and quantities of hemoglobin extracted, especially considering that this procedure was developed to biomonitor and prove an exposure to sulfur mustard, and confirmed the results previously observed with non-spiked blood samples. Finally, the estimation of the hemoglobin loss as free globin α and globin β chains in the filtrate resulting from the filtration step on the 50 kDa cutoff Amicon® centrifugal device was in agreement with that observed during the development of the extraction procedure, since only 2.7% of the protein was loss on average for the nine samples treated.

3.3. Digestion on the trypsin IMER of the hemoglobin extracted from spiked blood samples and LC-MS/MS analysis of the resulting alkylated peptides

3.3.1. Optimization of the transfer volume between the injection loop and the IMER

In our previous study [16], the volume of the injection loop for sample injection was 5 µL. However, in order to improve the sensitivity of the LC-MS/MS procedure for the analysis of biological samples, the injection loop was replaced by a 20 µL loop. The volume of Tris-HCl solution used to transfer the hemoglobin from the injection loop to the trypsin IMER, which had been optimized in our previous study for an injection loop of 5 µL, was thus optimized for this new set up (**Fig.S2**).

Taking into account the volume of the injection loop (20 µL), the volume of the connection tubes between the loop and the trypsin IMER (4.4 µL) and the IMER volume (44 µL), the transfer volume must range between 24.4 µL,

volume from which hemoglobin is totally transferred into the IMER, and 68.4 µL, volume from which the hemoglobin solution exceed the IMER void volume without being digested. The IMER volume (44 µL) was estimated by multiplying the geometrical pre-column volume (62.8 µL) by a theoretical porosity of 0.7. Knowing that the optimal transfer volume determined for a 5 µL injection loop was 40 µL and that it had been demonstrated with an ANOVA test that the transfer volume had no significant effect on the alkylated peptides peak areas [16], only two transfer volumes (40 and 50 µL) were compared for the new 20 µL injection loop, by digesting 20 µg of standard hemoglobin in TRIS-HCl buffer (1 mg. mL^{-1}), previously exposed to sulfur mustard at 100 $\mu g.mL^{-1}$ in pure water, in *stop flow* during 30 min and at 25°C. For each transfer volume tested, three independent digestions were carried out in the same conditions (n=3). The influence of the transfer volume on the peak areas corresponding to the alkylated peptides is presented on the **Fig.S7A** and **Fig.S7B** for globin α and globin β, respectively. A Student test (α=0.05) was performed in order to determine if the transfer volume had a significant effect on the peak areas and the results have demonstrated that there was no significant difference between the two volumes studied. Nevertheless, it appeared that a transfer volume of 50 µL led to more intense peak areas for 65.6% of the MRM transitions studied and that the results obtained were more repeatable than those obtained with a transfer volume of 40 µL for 50% of the MRM transitions. Moreover, the RSD were inferior to 10% for more than 69% of the MRM transitions with a transfer volume of 50 µL. Consequently, a transfer volume of 50 µL was considered as optimal for this new set up with an injection loop of 20 µL and was chosen for the digestion on the trypsin IMER of hemoglobin extracted from blood samples exposed to sulfur mustard.

3.3.2. Digestion on IMER of the hemoglobin extracted from spiked blood samples

Hemoglobin was extracted from whole blood samples exposed to different concentration levels of sulfur mustard (0.014 $\mu g.mL^{-1}$, 0.07 $\mu g.mL^{-1}$, 0.14 $\mu g.mL^{-1}$, 0.70 $\mu g.mL^{-1}$, 1.4 $\mu g.mL^{-1}$, 5.6 $\mu g.mL^{-1}$ and 28 $\mu g.mL^{-1}$) and digested on the laboratory-made trypsin IMER, using the protocol mentioned in the section 2.5. For hemoglobin extracted once from each of the seven sulfur mustard concentrations studied, three independent digestions were performed in the same conditions and on the same IMER since our previous study [16] proved that the IMERs were reusable. Based on the extracted hemoglobin concentrations estimated with the BCA assay for each blood sample exposed to sulfur mustard, solutions corresponding to 1 $mg.mL^{-1}$ of hemoglobin were prepared for each sulfur mustard concentration by diluting the extracted hemoglobin with an adequate volume of TRIS-HCl buffer (pH 8.0). 20 µL of the prepared solutions, *i.e.* 20 µg of hemoglobin, were then digested on the trypsin IMER and the resulting peptides were transferred to the trap column then the analytical column. Only the alkylated peptides were detected by mass spectrometry in MRM mode (see **Table S1** for mass parameters and transitions). An example of MRM chromatograms obtained for the 13 alkylated peptides resulting from the tryptic digestion of hemoglobin extracted from whole blood exposed to sulfur mustard at 0.7 $\mu g.mL^{-1}$ is presented in **Fig.2A**. As a comparison, the chromatogram obtained for standard hemoglobin (50 $mg.mL^{-1}$) *in vitro* exposed to an equivalent concentration of sulfur mustard (i.e. 0.25 $\mu g.mL^{-1}$[16]) in pure water is given in **Fig.2B**. If the MRM chromatograms resulting from the analysis of the biological sample are as clean as the one obtained in pure media, thus indicating that there are no matrix effects that could lead to an increased background noise, it was also observed that the peak intensities greatly varied between the two media, especially for the peptides α-T9 + HETE, β-T1 + HETE, β-9 + HETE and β-11 + HETE, which suggests that hemoglobin adduction by sulfur mustard depends on the matrix.

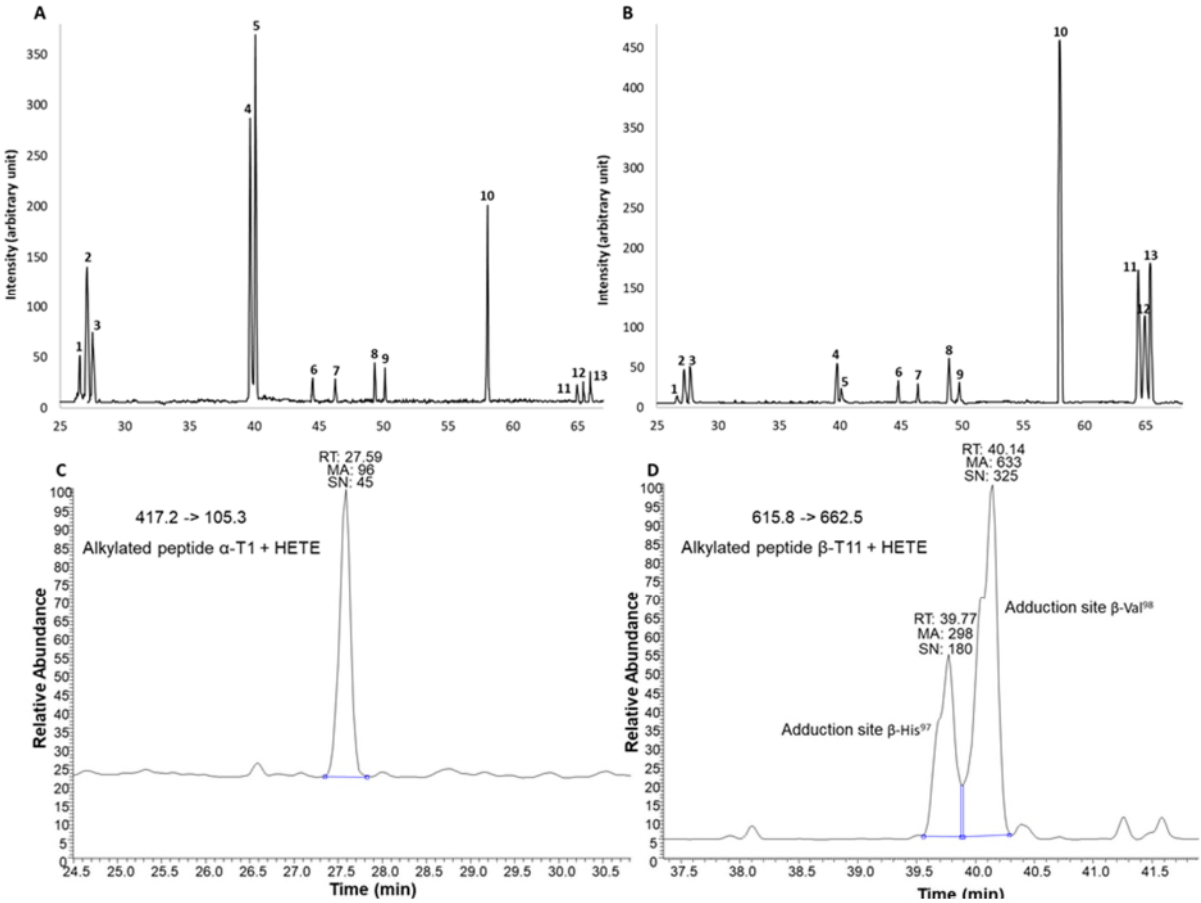

Fig.2. Overlay of MRM chromatograms corresponding to the alkylated peptides resulting from the tryptic digestion on IMER 1 (*stop flow* during 30 min at 25°C) of hemoglobin extracted from whole blood *in vitro* exposed to sulfur mustard at 0.7 µg.mL^{-1} (**A**) or of standard hemoglobin *in vitro* exposed to sulfur mustard at 0.25 µg.mL^{-1} in pure water (**B**). Peak 1: β-T1 + HETE (adduction site β-His^{2}), peak 2: β-T1 + HETE (adduction site β-Val^{1}), peak 3: α-T1 + HETE (adduction site α-Val^{1}), peak 4: β-T11 + HETE (adduction site β-His^{97}), peak 5: β-T11 + HETE (adduction site β-Val^{98}), peak 6: β-T3 + HETE (adduction site β-Glu^{22}), peak 7: β-T3 + HETE (adduction site β-Glu^{26}), peak 8: α-T6 + HETE (adduction site α-His^{45}), peak 9: α-T6 + HETE (adduction site α-His^{50}), peak 10: β-T9 + HETE (adduction site β-His^{77}), peak 11: α-T9 + HETE (adduction site α-His^{87}), peak 12: α-T9 + HETE (adduction site α-His^{89}), peak13 : α-T9 + HETE (adduction site α-His^{72}). MRM chromatograms of the alkylated peptide α-T1 + HETE (adduction site α-Val^{1}) (**C**) and the alkylated peptide β-T11 + HETE alkylated on the residue β-His^{97} (t_R=39.77 min) or the residue β-Val^{98} (t_R=40.14 min) (**D**) obtained after extraction of hemoglobin from whole blood exposed to 0.014 µg.mL^{-1} of sulfur mustard and its digestion on trypsin IMER during 30 min at 25°C, followed by LC-MS/MS analysis.

The main objective of these analyses was to check if the linear link between the sulfur mustard concentration and the alkylation degree of hemoglobin, previously observed in the case of studies conducted on hemoglobin standards exposed to sulfur mustard in pure water, was also observed in the biological samples as the extraction method from blood is now available. Therefore, the evolution of the peak areas of alkylated peptides (previously identified [13]) as a function of the sulfur mustard concentration was studied and the results obtained are reported in **Table 3** for all the alkylated peptides and illustrated in **Fig.3** for the alkylated peptide α-T1 + HETE, taken as an example. As seen on this figure, a satisfying linearity was observed on the concentration range studied for the two MRM transitions corresponding to this peptide. This linearity was also observed for the whole sulfur mustard concentration range (0.014-28 µg.mL^{-1}) studied for all the other alkylated peptides (see **Table 3** for the most

intense MRM transition of each peptide and **Table S2** for all the MRM transitions), thus confirming the results obtained for purified hemoglobin in pure water [16]. Moreover, it is important to note that the RSD observed for the peak areas corresponding to the most intense MRM transitions were inferior to 10% in 58% of the cases and globally ranged from 0.1 to 46.2%, hence highlighting the repeatability of the entire treatment and analytical procedure applied to whole blood samples, including the extraction step of hemoglobin, the digestion on IMER and the LC-MS/MS analysis of the resulting alkylated peptides.

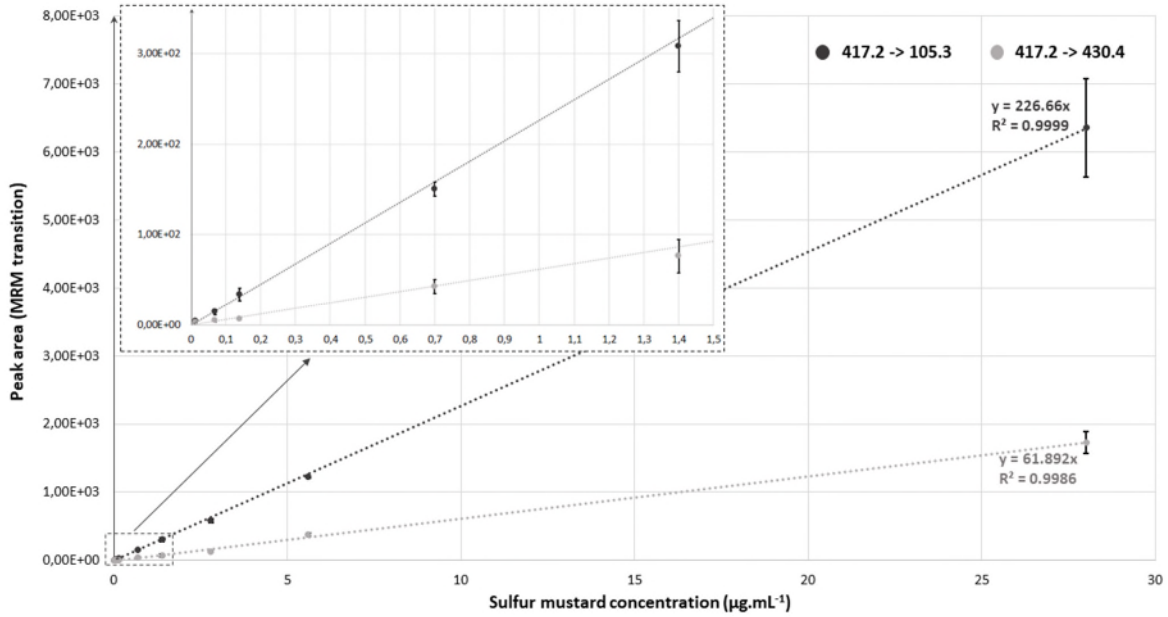

Fig.3. Evolution of the peak area corresponding to the alkylated peptide α-T1 + HETE (adduction site α-Val[1]) with the sulfur mustard concentration (n=3, 3 independent digestions performed on the IMER for each sulfur mustard concentration).

Experimentally, the digestion on the trypsin IMER coupled on-line with the LC-MS/MS analysis allowed the detection of alkylated peptides resulting from the exposure of blood to sulfur mustard at a concentration as low as 0.014 µg.mL^{-1}, which is similar to a previous study carried out by Noort *et al.* who were able to detect the adducts formed with the N-terminal valine residues of hemoglobin by GC-MS after in vitro exposure of blood samples with sulfur mustard at 15.9 ng.mL^{-1}, using a modified Edman degradation procedure [15]. However, it must be noted that the concentration of 0.014 µg.mL^{-1} was the lowest sulfur mustard exposure dose that was provided by the DGA CBRN Defense and that it could surely be possible to detect the alkylated peptides resulting from the exposure of whole blood to concentrations five to ten times inferior. Examples of extracted ion chromatograms (EIC) corresponding to the most intense MRM transitions of the alkylated peptides α-T1 + HETE and β-T11 + HETE obtained for a concentration of sulfur mustard of 0.014 µg.mL^{-1} are presented in **Fig.2C** and **Fig.2D**, respectively. Based on the S/N ratios experimentally obtained for the lowest concentrations of sulfur mustard (0.014 and 0.07 µg.mL^{-1}), it was possible to estimate the sensitivity of the whole treatment and analytical procedure. Thus, the estimation of the theoretical LOD and LOQ, *i.e.* the lowest concentrations of sulfur mustard that could respectively be detected and quantified with the developed analytical procedure, for the most intense MRM transition of each alkylated peptide is reported in the **Table 3**. As seen in this table, depending on the targeted peptides, it would theoretically be possible to detect an exposure of whole blood to a concentration of

sulfur mustard inferior to 1 ng.mL^{-1}, which highlights the great sensitivity of the analytical procedure. Therefore, the alkylated peptide β-T11 + HETE could be used to sensitively detect an exposure to sulfur mustard to concentrations down to 0.1 ng.mL^{-1}, while the peptide β-T9 + HETE could be used as confirmation of exposure.

Finally, the alkylation rates, corresponding to the alkylated peptide/sum of alkylated peptides ratio and illustrating the part of the total alkylation induced on hemoglobin upon exposure to sulfur mustard represented by each peptide, was determined after tryptic digestion on IMER of the hemoglobin extracted from blood samples exposed to sulfur mustard. This ratio was calculated for all the alkylated peptides for the seven concentrations of sulfur mustard studied. These ratios, reported in the **Table 3**, demonstrated that the alkylation rates induced on hemoglobin were independent of the sulfur mustard concentration, with RSD ranging from 3.4 to 10.6%. However, when comparing the results obtained for the analysis of biological samples with those obtained in pure water [16], major differences could be observed (see **Table 3**), confirming the differences observed on the chromatograms of both matrices (see **Fig.2A** and **Fig.2B**). Indeed, in blood samples, it seems that most of the sulfur mustard binds with the adduction sites of globin β, since the 7 alkylation sites localized on the globin β chains represent more than 85.9% of the total adduction induced on hemoglobin. The residues β-His^{77}, β-His^{97} and β-Val^{98} alone represent more than 72% of the alkylation. Therefore, if, in pure water, the preferential adduction sites were the histidine residues of both globin α and globin β [16], the two adduction sites of the peptide β-T11 are the most alkylated sites in the biological matrix. Thus, the residues β-His^{97} and β-Val^{98} are respectively 5.7 and 22.3 times more alkylated in blood samples than in pure water. These observations indicate that the adduction of sulfur mustard on hemoglobin in whole blood is very different from that occurring in pure water, probably due to a modification of the hemoglobin structure in the biological matrix and thus, to a variability of the access of sulfur mustard to the different adduction sites of the protein. Finally, it appeared that the alkylation on the N-terminal valine residues α-Val^{1} and β-Val^{1}, adduction sites preferentially targeted with the modified Edman degradation procedure, respectively represented 4.16 and 9.60% of the total alkylation of hemoglobin in whole blood, thus highlighting the importance of the newly developed analytical method, targeting several alkylation sites at once, as an alternative to the current procedure.

Conclusion

In this study, a new procedure was developed and optimized for the extraction of hemoglobin from whole blood samples. This procedure includes the hemolysis of the isolated RBCs by two freezing cycles of 1 h at -80°C followed by the purification of the resulting lysate by ultrafiltration on Amicon® 100 kDa and 50 kDa centrifugal devices. This simple method was applied to the extraction of hemoglobin from blood samples spiked with sulfur mustard. In addition to being repeatable (RSD=12.6 %), the optimized procedure allowed to extract more than 75% of the hemoglobin theoretically present in the initial blood samples treated.

After fast digestion on a trypsin IMER of the hemoglobin extracted from blood samples exposed to sulfur mustard at concentrations ranging between 0.014 and 28 µg.mL^{-1}, it was confirmed that a linear link existed between the sulfur mustard concentration and the peak areas of all the alkylated peptides. It implied that this method could be used for semi-quantitative analysis in further studies. A satisfying sensibility was also obtained since, depending on the adduction sites, it could theoretically be possible to detect peptides resulting from the exposure of whole blood samples to a concentration of sulfur mustard down to 1 ng.mL^{-1}. The entire analytical method presented in this study (sample treatment + IMER digestion + LC-MS/MS analysis of the peptides) has a duration of 7 hours, which makes it a great alternative to the modified Edman degradation procedure, for which the sample treatment alone lasts more than 1 day in the laboratory of the French Defense Procurement Agency, while presenting a

similar sensitivity. Moreover, the sample treatments steps, *i.e.* the RBCs hemolysis and the lysate filtration, can be performed simultaneously for several blood samples, thus reducing the time required for the analysis of multiple samples.

Finally, the results obtained in this study highlighted important differences regarding the preferential adduction sites of sulfur mustard on hemoglobin, between the exposition of purified hemoglobin in pure water and those in blood samples. It was observed that, for biological samples, the main alkylation sites were the residues β-His^{97} and β-Val^{98}, representing almost 53% of the total adduction of the protein, against only 5.7% on purified hemoglobin in water. These results showed that the alkylation of hemoglobin by sulfur mustard greatly varies depending on the matrix, hence suggesting interaction differences with the protein, whose structure may vary depending on its environment. The whole developed and optimized procedure has yet to be applied to the analysis of blood samples resulting from the *in vivo* exposure of casualties to sulfur mustard, and collected on various days following the exposure, in order to determine the detection time-frame of the alkylated peptides.

Compliance with ethical standards

This is not a clinical study on humans/animals. Human blood samples from healthy individuals (batches 10200088369 and 1021002035) were provided by the Centre de Transfusion des Armées (CTSA).

Conflict of interest

The authors declare that they have no conflict of interest.

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[18] C.T. Andrade, L.A.M. Barros, M.C.P. Lima, E.G. Azero, Purification and characterization of human hemoglobin: effect of the hemolysis conditions, Int. J. Biol. Macromol. 34 (2004) 233–240. https://doi.org/10.1016/j.ijbiomac.2004.05.003.

Table 1. Quantities of hemoglobin extracted from non-spiked whole blood samples after hemolysis of the RBCs using two different techniques or number of cycles, and further filtration and purification of the resulting lysates with two types of 100 kDa cutoff filters. The amounts of hemoglobin were estimated using a colorimetric BCA assay. RT: room temperature.

Whole blood sample treated		Assay 1 (see Fig.S3)		Assay 2 (See Fig.S4)		Assay 3 (see Fig.1)	
Initial sample volume		4 mL		4 mL		2 mL	
RBCs hemolysis		Sonication (5 x 1 min, 130W)	Freezing at -80°C during 1h	Freezing at -80°C during 1h		Freezing at -80°C during 2h in two cycles of 1h	Freezing at -80°C during 2h in one cycle
Lysate centrifugation		4,500 rpm 4°C 10 min		6,400 rpm RT 5 min		6,400 rpm RT 5 min	
Lysate purification		Ultrafiltration on Amicon® 100kDa (4,500 rpm 4°C 10 min) and 50kDa cutoff filters (4,500 rpm 4°C 10 min)		Ultrafiltration on Amicon®100kDa (4,500 rpm 4°C 10 min, x3) and 50kDa cutoff filters (4,500 rpm 4°C 10 min, x3)	Ultrafiltration on Centrisart® 100kDa (4,500 rpm 4°C 10 min, x6) and Amicon® 50kDa cutoff filters (4,500 rpm 4°C 10 min, x3)	Ultrafiltration on Amicon® 100kDa (4,500 rpm 4°C 10 min, x3) and 50kDa cutoff filters (4,500 rpm 4°C 10 min, x3)	
Quantity of hemoglobin extracted	**Replicate 1**	58.6 mg	96.2 mg	104.1 mg	20.5 mg	94.9 mg	80.6 mg
	Replicate 2	35.9 mg	102.4 mg	90.3 mg	17.7 mg	101.7 mg	87.0 mg
	Replicate 3	109.5 mg	97.8 mg	95.3 mg	18.5 mg	-[b]	-[b]
Mean quantity of Hb extracted (RSD)		68.0 mg (45.2%)	98.8 mg (2.7%)	95.6 mg (5.7%)	18.9 mg (6.2%)	94.9-101.7 mg	80.6-87.0 mg
Mean percentage of Hb extracted		22.7%[a]	32.9%[a]	31.9%[a]	6.3%[a]	63.3-67.8%[a]	53.7-58.0%[a]

[a] Considering an average concentration of hemoglobin in human blood of 150 mg.mL^{-1} (usual range:120 to 180 mg.mL^{-1}) ; [b] Third replicate not performed

Table 2. Quantities of hemoglobin extracted from whole blood samples (1 mL) exposed to different concentrations of sulfur mustard (HD) and submitted to hemolysis of the RBCs by freezing at -80°C during 1h repeated twice, followed by the filtration and purification of the resulting lysates. The amounts of hemoglobin were estimated using a colorimetric BCA assay.

Samples	Quantity of Hb extracted from whole blood	Percentage of Hb extracted from whole blood[a]	Quantity of Hb lost after filtration with Amicon® 50 kDa cutoff filter	Percentage of Hb lost after filtration with Amicon® 50 kDa cutoff filter[a]
Unspiked whole blood	124.4 mg	82.9%	4.0 mg	2.7%
Unspiked whole blood + 1% iPrOH	127.7 mg	85.1%	4.3 mg	2.9%
Whole blood + HD 0.014 µg.mL^{-1}	121.4 mg	80.9%	4.3 mg	2.9%
Whole blood + HD 0.07 µg.mL^{-1}	82.3 mg	54.9%	5.0 mg	3.3%
Whole blood + HD 0.14 µg.mL^{-1}	121.5 mg	81.0%	3.8 mg	2.5%
Whole blood + HD 0.7 µg.mL^{-1}	112.3 mg	74.9%	3.6 mg	2.4%
Whole blood + HD 1.4 µg.mL^{-1}	96.7 mg	64.5%	3.4 mg	2.3%
Whole blood + HD 5.6 µg.mL^{-1}	115.9 mg	72.3%	4.9 mg	3.3%
Whole blood + HD 28 µg.mL^{-1}	126.2 mg	84.1%	3.5 mg	2.3%
Mean (RSD, n=9)	114.3 mg (12.6%)	75.6% (12.7%)	4.1 mg (13.2%)	2.7% (13.6%)

[a] Considering an average concentration of hemoglobin in human blood of 150 mg.mL^{-1} (usual range: 120-180 mg.mL^{-1})

Table 3. Linear regression equations and coefficient values of the regression curves obtained for the evolution of the peak area corresponding to each alkylated peptide resulting from the tryptic digestion of hemoglobin extracted once from whole blood samples incubated with sulfur mustard vs the sulfur mustard concentration, minimal concentration of *in vitro* exposure to sulfur mustard that can be detected (LOD) or quantified (LOQ) in whole blood samples and comparison of the mean alkylation rates obtained in the biological matrix or in pure water. Blood samples were incubated *in vitro* with seven sulfur mustard concentration levels (0.014 µg.mL^{-1}, 0.07 µg.mL^{-1}, 0.14 µg.mL^{-1}, 0.7 µg.mL^{-1}, 1.4 µg.mL^{-1}, 5.6 µg.mL^{-1} and 28 µg.mL^{-1}) while lyophilized human hemoglobin in pure water was incubated in vitro at five sulfur mustard concentration levels (0.25 µg.mL^{-1}, 0.5 µg.mL^{-1}, 1 µg.mL^{-1}, 10 µg.mL^{-1} and 100 µg.mL^{-1}). Three independent digestions were performed on the trypsin IMER (digestion in stop flow at 25°C during 30 min) for each sulfur mustard concentration (n=21 for blood samples and n=15 in pure water).

Alkylated peptide	Adduction site	MRM transition (quantitative ion)	Linear regression equation and coefficient value of the regression curve	Estimated sensitivity in whole blood samples		Mean alkylation rate (alkylated peptide/total of alkylated peptides)[a] (RSD)	
				LOD (ng.mL^{-1}) S/N=3	LOQ (ng.mL^{-1}) S/N=10	Blood sample (n=21)	Pure water (n=15) [16]
α-T1 + HETE	α-Val^{1}	417.2 -> 105.3	y = 226.84x R² = 0.9999	0.9	3.1	4.16% (5.9%)	3.71% (10.9%)
α-T6 + HETE	α-His^{45}	646.7 -> 237.0	y = 98.108x R² = 0.9991	1.1	3.5	1.80% (8.1%)	4.80% (7.5%)
α-T6 + HETE	α-His^{50}	646.7 -> 237.0	y = 59.603x R² = 0.9993	1.8	5.8	1.16% (9.5%)	2.47% (8.8%)
α-T9 + HETE	α-His^{72}	776.0 -> 977.8	y = 135.30x R² = 0.9999	1.6	5.2	2.70% (7.9%)	14.45% (9.0%)
α-T9 + HETE	α-His^{87}	776.0 -> 977.8	y = 108.80x R² = 0.9999	1.3	4.4	1.92% (8.6%)	13.82% (9.4%)
α-T9 + HETE	α-His^{89}	776.0 -> 977.8	y = 133.35x R² = 0.9999	1.9	6.4	2.27% (15.9%)	9.07% (2.9%)
β-T1 + HETE	β-Val^{1}	529.0 -> 105.0	y = 505.11x R² = 0.9997	1.0	3.3	9.60% (3.4%)	3.47% (3.2%)
β-T1 + HETE	β-His^{2}	529.0 -> 105.0	y = 108.26x R² = 0.9999	16.2	53.9	2.27% (10.4%)	0.83% (7.2%)
β-T3 + HETE	β-Glu^{22}	710.0 -> 758.6	y = 38.801x R² = 0.9946	3.2	10.8	0.86% (10.6%)	2.29% (8.7%)
β-T3 + HETE	β-Glu^{26}	710.0 -> 763.3	y = 41.148x R² = 0.9959	2.8	9.3	0.87% (9.3%)	2.01% (7.8%)
β-T9 + HETE	β-His^{77}	592.0 -> 781.5	y = 988.45x R² = 0.9996	0.3	1.1	19.33% (7.0%)	36.64% (5.8%)
β-T11 + HETE	β-His^{97}	615.8 -> 662.5	y = 1223.8x R² = 0.9991	0.2	0.8	23.47% (5.7%)	4.10% (9.8%)
β-T11 + HETE	β-Val^{98}	615.8 -> 662.5	y = 1579.9x R² = 0.9996	0.1	0.4	29.60% (5.3%)	1.33% (5.7%)

[a] Alkylation rates calculated considering only the most intense MRM transition for each alkylated peptide

Supplementary material

Analysis of long-lived sulfur mustard-human hemoglobin adducts in blood samples by red blood cells lysis and on-line coupling of digestion on an immobilized-trypsin reactor with liquid chromatography-tandem mass spectrometry

Florine Hallez[1], Audrey Combès[1], Charlotte Desoubries[2], Anne Bossée[2], Valérie Pichon[1,3,*]

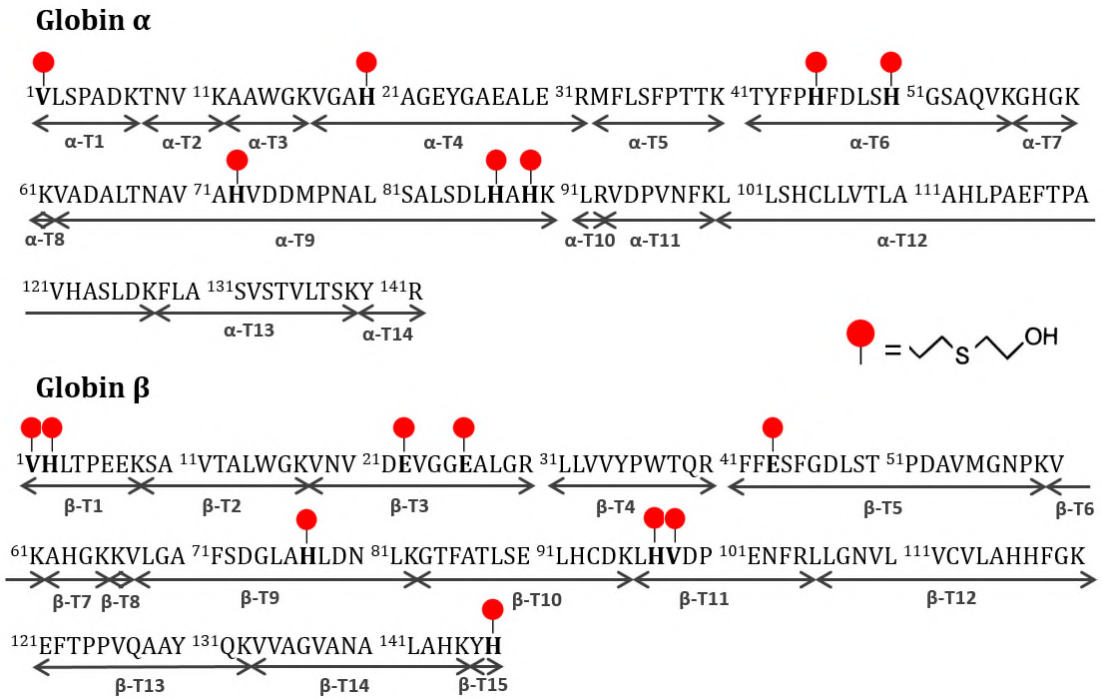

Fig.S1. Localization of the alkylation sites (red circles) of human hemoglobin by sulfur mustard (HD) identified LC-ESI$^+$-MS/MS analysis of tryptic digests [11-13].

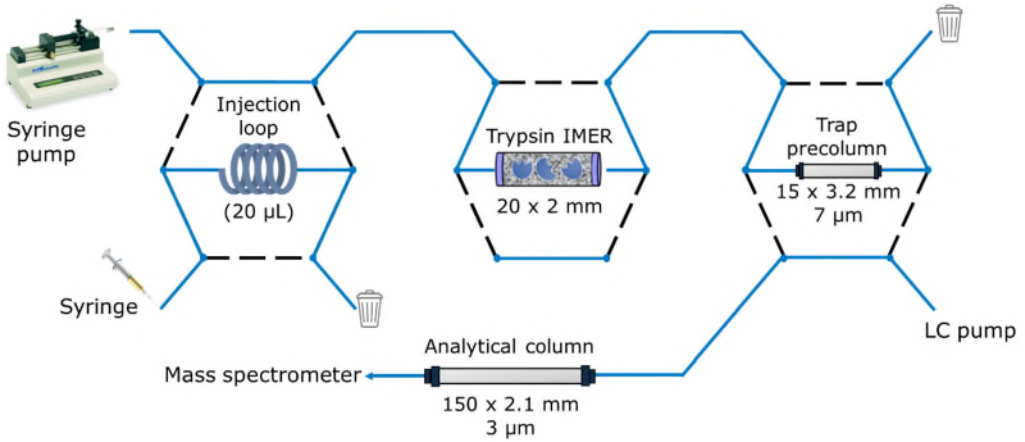

Fig.S2. Experimental set-up used for the on-line digestion on the trypsin IMER of alkylated hemoglobin and LC-MS/MS analysis of the resulting peptides.

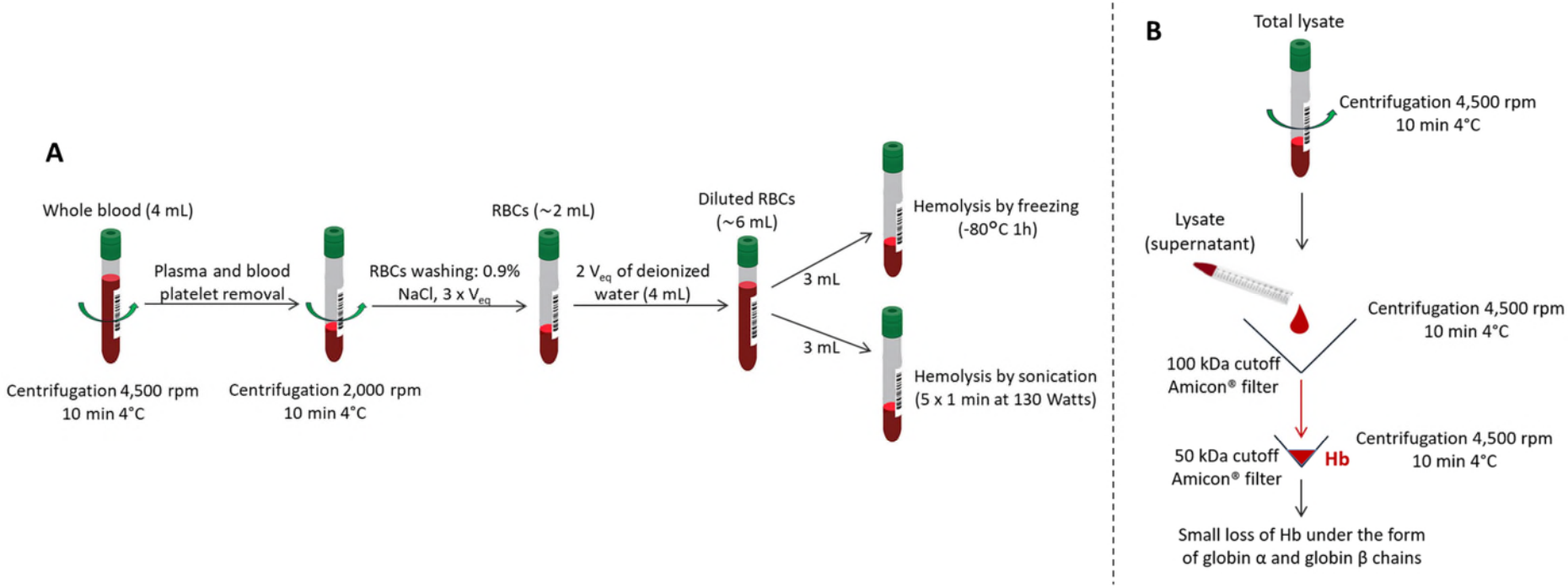

Fig.S3. Schematic representation of the isolation of RBCs from whole blood and their hemolysis by freezing at -80°C during 1 hour or by sonication (**A**) followed by the lysate filtration and purification of the extracted hemoglobin by ultrafiltration on 100 and 50 kDa cutoff Amicon® filters (**B**). V_{eq}: equivalent volume. See assay 1 in Table 1 for experimental values of extracted Hb obtained with both hemolysis methods.

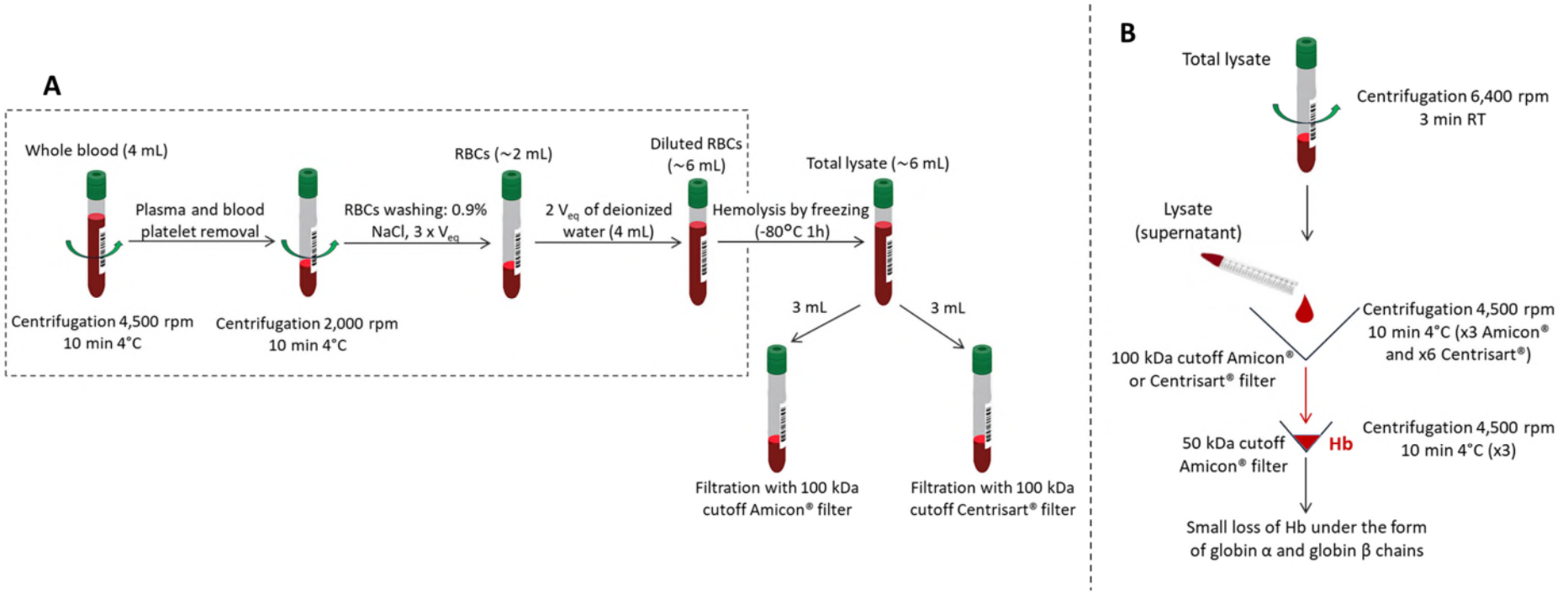

Fig.S4. Schematic representation of the isolation of RBCs from whole blood and their hemolysis by freezing at -80°C during 1 hour (**A**) followed of the lysate filtration and purification of the extracted hemoglobin using 100 kDa cutoff Amicon® or Centrisart® filters (**B**). The steps identical to those previously described for the hemolysis procedure are framed. V_{eq}: equivalent volume, RT: room temperature. See assay 2 in Table 1 for experimental values of extracted Hb obtained with both 100 kD cutoff filter.

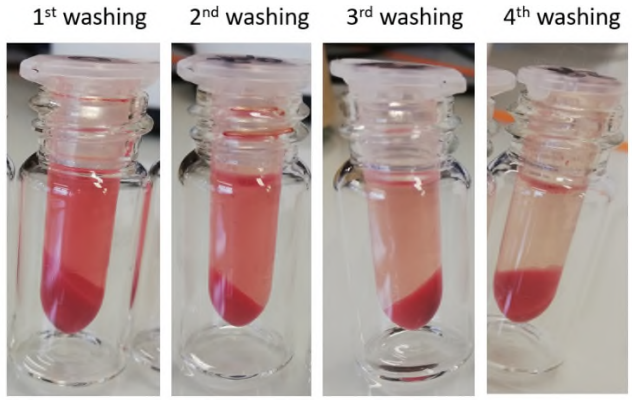

Fig.S5. Washing with pure water by centrifugation at 6,400 rpm of the cell debris and the non-lysed RBCs resulting from two cycles of lysis of the RBCs by freezing at -80°C during 1h.

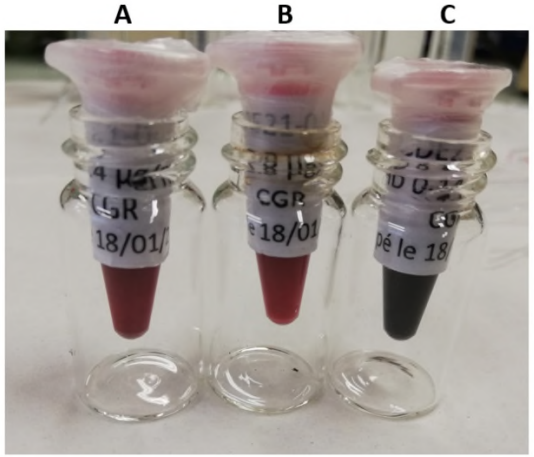

Fig.S6. Highlighting of the color difference between the samples of whole blood spiked with sulfur mustard at a concentration of 1.4 µg.mL^{-1} (A) and 0.07 µg.mL^{-1} (B) and the sample of blood spiked with sulfur mustard at a concentration of 0.14 µg.mL^{-1} (C), after addition of 1 mL of deionized water to the washed RBCs.

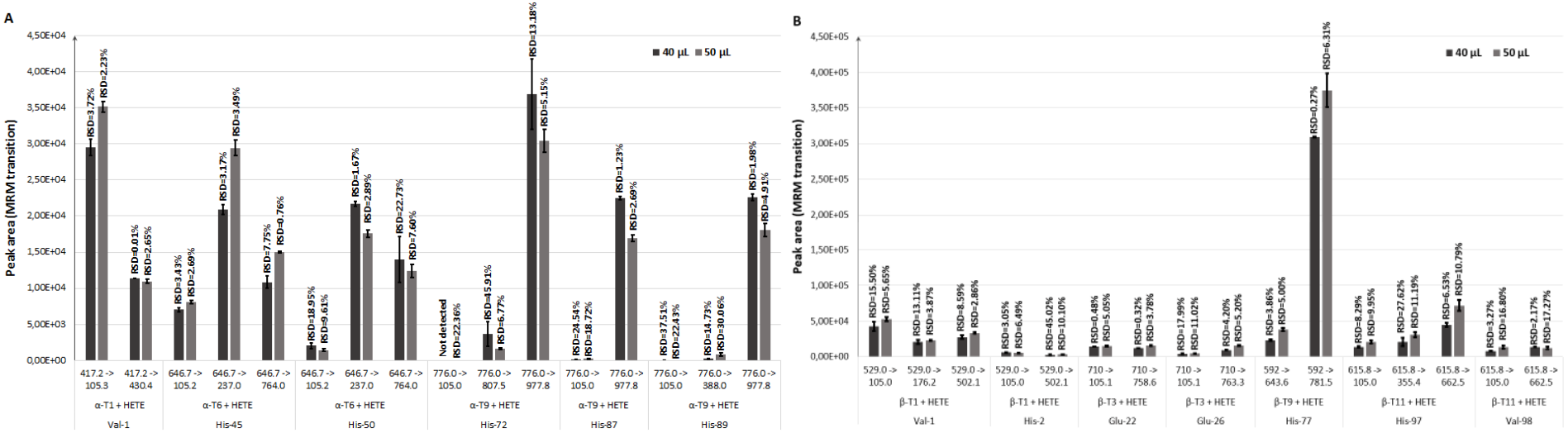

Fig.S7. Influence of the transfer volume between the 20µL injection loop and the trypsin IMER on the peak area corresponding to the alkylated peptides of globin α (**A**) and globin β (**B**) resulting from the digestion of standard hemoglobin exposed to sulfur mustard at 100 µg.mL^{-1}. n=3 (3 independent digestions performed in *stop flow* on IMER during 30 min at 25°C for each transfer volume).

1

Table S1. Mass parameters and transitions used for the analysis in MRM mode of the peptides resulting from the digestion on trypsin IMERs of human hemoglobin (20 µg) extracted from whole blood samples incubated *in vitro* with sulfur mustard. The HD adduction sites are indicated in bold in the peptide sequences.

Time segment	Peptide	Precursor ion	Tube lens	Product ions	Corresponding peptide fragment	Collision energy
0-25 min	α-T1 VLSPADK (1-7)	m/z 365.2 (2^+)	70 V	m/z 517.2	SPADK (y_5^+)	15 eV
				m/z 630.3	LSPADK (y_6^+)	15 eV
24-35 min	α-T1 **V**LSPADK (1-7) + HETE	m/z 417.2 (2^+)	95 V	m/z 105.3	$HETE^+$	15 eV
				m/z 430.4	PADK (y_4^+)	15 eV
	β-T1 **V**HLTPEEK (1-8) + HETE	m/z 529.0 (2^+)	90 V	m/z 105.0	$HETE^+$	25 eV
				m/z 176.2	**V** (a_1^+) + HETE*	25 eV
				m/z 502.1	PEEK (y_4^+)	25 eV
	β-T1 V**H**LTPEEK (1-8) + HETE	m/z 529.0 (2^+)	90 V	m/z 105.0	$HETE^+$	25 eV
				m/z 502.1	PEEK (y_4^+)	25 eV
35-42 min	β-T11 L**H**VDPENFR (96-104) + HETE	m/z 615.8 (2^+)	115 V	m/z 105.0	$HETE^+$	25 eV
				m/z 355.4	L**H** (b_2^+) + HETE*	25 eV
				m/z 662.5	PENFR (y_5^+)	25 eV
	β-T11 LH**V**DPENFR (96-104) + HETE	m/z 615.8 (2^+)	115 V	m/z 105.0	$HETE^+$	25 eV
				m/z 662.5	PENFR (y_5^+)	25 eV
42-47.5 min	β-T3 VNVD**E**VGGEALGR (18-30) + HETE	m/z 710.0 (2^+)	100 V	m/z 105.1	$HETE^+$	20 eV
				m/z 758.6	VGGEALGR (y_8^+)*	25 eV
	β-T3 VNVDEVGG**E**ALGR (18-30) + HETE	m/z 710.0 (2^+)	100 V	m/z 105.1	$HETE^+$	20 eV
				m/z 763.3	GG**E**ALGR (y_7^+) + HETE*	20 eV
47.5-57 min	α-T6 TYFP**H**FDLSHGSAQVK (41-56) + HETE	m/z 646.7 (3^+)	125 V	m/z 105.2	$HETE^+$	30 eV
				m/z 237.0	TY (a_2^+)	30 eV
				m/z 764.0	P**H**FDLSHGSAQVK (y_{13}^{2+}) + HETE	25 eV
	α-T6 TYFPHFDLS**H**GSAQVK (41-56) + HETE	m/z 646.7 (3^+)	125 V	m/z 105.2	$HETE^+$	30 eV
				m/z 237.0	TY (a_2^+)	30 eV
				m/z 764.0	PHFDLS**H**GSAQVK (y_{13}^{2+}) + HETE	25 eV
57-62 min	β-T9 VLGAFSDGLA**H**LDNLK (67-82) + HETE	m/z 592.0 (3^+)	90 V	m/z 643.6	SDGLA**H**LDNLK (y_{11}^{2+}) + HETE	20 eV
				m/z 781.5	GAFSDGLA**H**LDNLK (y_{14}^{2+}) + HETE	15 eV
63-99 min	α-T9 VADALTNAVA**H**VDDMPNALSALSDLHAHK (62-90) + HETE	m/z 776.0 (4^+)	125 V	m/z 105.0	$HETE^+$	25 eV
				m/z 807.5	SDLHAHK (y_7^+)	25 eV
				m/z 977.8	DALTNAVA**H**VDDMPNALSALSDLHAHK (y_{27}^{3+}) + HETE	20 eV
		m/z 776.0	125 V	m/z 105.0	$HETE^+$	25 eV

	α-T9 VADALTNAVAHVDDMPNALSA LSDL**H**AHK (62-90) + HETE	(4^+)		m/z 977.8	DALTNAVAHVDDMPNALSALSDL**H**AHK (y_{27}^{3+}) + HETE	20 eV
	α-T9 VADALTNAVAHVDDMPNALSA LSDLHA**H**K (62-90) + HETE	m/z 776.0 (4^+)	125 V	m/z 105.0	$HETE^+$	25 eV
				m/z 388.0	**H**K (y_2^+) + HETE*	25 eV
				m/z 977.8	DALTNAVAHVDDMPNALSALSDLHA**H**K (y_{27}^{3+}) + HETE	20 eV

Table S2. Linear regression equations and coefficient values of the regression curves obtained for the evolution of the peak area corresponding to each alkylated peptide resulting from the tryptic digestion of hemoglobin extracted from whole blood samples exposed to sulfur mustard vs the sulfur mustard concentration, and ratios of the MRM transition intensities for each alkylated peptide. Blood samples were incubated *in vitro* with seven sulfur mustard concentrations (0.014 µg.mL^{-1}, 0.07 µg.mL^{-1}, 0.14 µg.mL^{-1}, 0.7 µg.mL^{-1}, 1.4 µg.mL^{-1}, 5.6 µg.mL^{-1} and 28 µg.mL^{-1}). Three independent digestions of the blood extract obtained once for each of the seven sulfur mustard concentrations were performed (n=21) on the trypsin IMER (digestion in *stop flow* at 25°C during 30 min with a transfer volume of 50 µL).

Alkylated peptide	Adduction site	MRM transition	Linear regression equation and coefficient value of the regression curve	MRM transition intensities ratio[a]
α-T1 + HETE	α-Val^{1}	417.2 -> 105.3	y = 226.84x R² = 0.9999	1
		417.2 -> 430.4	y = 62.038x R² = 0.9994	0.27
α-T6 + HETE	α-His^{45}	646.7 -> 105.2	y = 22.152x R² = 0.9981	0.23
		646.7 -> 237.0	y = 98.108x R² = 0.9991	1
		646.7 -> 764.0	y = 42.262x R² = 0.9984	0.43
α-T6 + HETE	α-His^{50}	646.7 -> 105.2	y = 5.6286x R² = 0.9871	0.09
		646.7 -> 237.0	y = 59.603x R² = 0.9993	1
		646.7 -> 764.0	y = 37.363x R² = 0.9991	0.63
α-T9 + HETE	α-His^{72}	776.0 -> 105.0	y = 1.7488x R² = 0.9873	0.01
		776.0 -> 807.5	y = 59.786x R² = 0.9976	0.44
		776.0 -> 977.8	y = 135.30x R² = 0.9999	1
α-T9 + HETE	α-His^{87}	776.0 -> 105.0	y = 2.7844x R² = 0.9998	0.03
		776.0 -> 977.8	y = 108.80x R² = 0.9999	1
α-T9 + HETE	α-His^{89}	776.0 -> 105.0	y = 1.7032x R² = 0.9958	0.01
		776.0 -> 388.0	y = 9.9341x R² = 0.9995	0.07
		776.0 -> 977.8	y = 133.35x R² = 0.9999	1
β-T1 + HETE	β-Val^{1}	529.0 -> 105.0	y = 505.11x R² = 0.9997	1
		529.0 -> 176.2	y = 268.57x R² = 0.9992	0.53
		529.0 -> 502.1	y = 346.08x R² = 0.9988	0.69
β-T1 + HETE	β-His^{2}	529.0 -> 105.0	y = 108.26x R² = 0.9999	1
		529.0 -> 502.1	y = 49.780x R² = 0.9961	0.46
β-T3 + HETE	β-Glu^{22}	710.0 -> 105.1	y = 34.756x R² = 0.9998	0.90
		710.0 -> 758.6	y = 38.801x R² = 0.9946	1
β-T3 + HETE	β-Glu^{26}	710.0 -> 105.1	y = 9.3711x R² = 0.9994	0.23
		710.0 -> 763.3	y = 41.148x R² = 0.9959	1
β-T9 + HETE	β-His^{77}	592.0 -> 643.6	y = 71.085x R² = 0.9894	0.07
		592.0 -> 781.5	y = 988.45x R² = 0.9996	1
β-T11 + HETE	β-His^{97}	615.8 -> 105.0	y = 339.94x R² = 0.9987	0.28

		615.8 -> 355.4	y = 776.57x R^2 = 0.9997	0.63
		615.8 -> 662.5	y = 1223.8x R^2 = 0.9991	1
β-T11 + HETE	β-Val^{98}	615.8 -> 105.0	y = 1150.9x R^2 = 0.9996	0.73
		615.8 -> 662.5	y = 1579.9x R^2 = 0.9996	1

[a] The ratios were calculated using the quantitative MRM transition, *i.e.* the most intense transition, as the denominator.