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Standard operation protocol for analysis of lipid hydroperoxides in human serum using a fully automated method based on solid-phase extraction and liquid chromatography–mass spectrometry in selected reaction monitoring

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ABSTRACT

Standard operating procedures (SOPs) are of paramount importance in the analytical field to ensure the reproducibility of the results obtained among laboratories. SOPs gain special interest when the aim is the analysis of potentially unstable compounds. An SOP for analysis of lipid hydroperoxides (HpETEs) is here reported after optimization of the critical steps to be considered in their analysis in human serum from sampling to final analysis. The method is based on automated hyphenation between solid-phase extraction (SPE) and liquid chromatography–mass spectrometry (LC–MS). The developed research involves: (i) optimization of the SPE and LC–MS steps with a proper synchronization; (ii) validation of the method—*viz.* accuracy study (estimated as 86.4% as minimum value), evaluation of sensitivity and precision, which ranged from 2.5 to 7.0 ng/mL (0.25–0.70 ng on column) as quantification limit and precision below 13.2%), and robustness study (reusability of the cartridge for 5 times without affecting the accuracy and precision of the method); (iii) stability study, involving freeze–thaw stability, short-term and long-term stability and stock solution stability tests. The results thus obtained allow minimizing both random and systematic variation of the metabolic profiles of the target compounds by correct application of the established protocol.

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1. Introduction

Sources of potential variability in metabolomics analysis can be ascribed to analytical or biological issues, the latter desirably correlated with physiological states or dietary, environmental, genetic or pathophysiological conditions. For this reason, metabolomics studies require in-depth validation in three progressive domains: (i) analytical issues, (ii) inter-/intra-personal biological variation, and (iii) correspondence with a given phenotype [1,2]. The control of the potential sources of variability is crucial to avoid errors in data interpretation.

Although metabolomics studies developed with different types of samples have revealed that biological variation is usually higher than that attributed to analytical variation, it is important to identify the main sources of analytical variability [3–5]. Thus, in addition to inter-individual and intra-individual variations, the sources of error in metabolomics are mainly associated to sampling and post-collection procedures such as freeze–thaw cycles or inadequate storage conditions [6]. It is well-known that unsuitable sampling and sample preparation protocols can lead to biased results owing anabolic or catabolic processes [7]. There is increased interest in rapid collection and handling of samples for metabolomics purposes, as turnover kinetics of some metabolites is known to be extremely fast. Accordingly, the time window between sampling and analysis should be as short as possible. For instance, many intracellular metabolites such as ATP and glucose-6-phosphate are extremely labile, with turnover rates of less than 2 s [8]. Thus, with identification purposes cellular metabolism must be stopped immediately upon sampling of the cells to prevent/minimize metabolite turnover. In fact, sample representation in metabolomics is only achieved when metabolism is efficiently interrupted during sampling by quenching. This step aims at instantaneous stop of metabolism by inhibiting the activity of endogenous enzymes. In this way, changes in the metabolic profile during

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sampling are suppressed. The common strategy for quenching is based on rapid modification of sample conditions, usually by a temperature shock [9].

Immediate analysis of the samples is not always possible and, therefore, sample storage is required (*e.g.* in banks of biological samples for research purposes). Sample storage is other critical source of error in metabolomics analysis. Most metabolites are preserved if samples are immediately frozen at temperature close to −80 °C (*e.g.* by liquid nitrogen). However, it is worth noting that differences in storage time or frequent thaw–freeze cycles may have a strong influence on the development of metabolomics models. Metabolic activity during sampling and storage requires stopping or minimizing changes in the metabolic profile either in concentration or structure. For this purpose, decreased temperatures during sample preparation (4 °C) and storage (−80 °C) are frequently implemented in analysis protocols [10,11]. For instance, appropriate storage and preparatory measures must immediately follow urine collection to ensure the quality of results in metabolomics analysis throughout the collection and analytical process. Thus, apart from samples storage at −80 °C, freeze–thaw cycles should be avoided whenever possible. The addition of a bacteriostatic preservative such as sodium azide can be crucial to avoid bacterial contamination [12].

The variability associated with sample storage or preliminary operations can be critical in targeted metabolomics approaches focused on a restricted set of metabolites. In this sense, the general trend in metabolomics goes towards standardization by development of high-throughput pipelines involving sequentially executed standard operating procedures (SOPs), each of which is designed to minimize both random and systematic variation of the target metabolic profiles. Furthermore, standardized reports enable researchers to compare and combine results from different studies. In the present research, an experimental planning has been designed to develop a standard protocol for metabolomics analysis of lipid hydroperoxides (HpETEs) in human serum. Fatty-acid hydroperoxides are the primary products of lipid peroxidation and the precursors for biosynthesis of HETEs and leukotrienes. For this reason, they have been selected as model compounds as their stability is seriously affected by inappropriate sampling and pretreatment protocols. The relevance of lipid hydroperoxides is supported by their role as key modulators of unsaturated fatty-acid oxygenation [13] or platelet aggregation [14–19], among others. Since HpETEs are rapidly reduced by cellular peroxidases to the corresponding hydroxyeicosatetraenoic acid (HETE) [20] a fast and precise method that improves the repeatability and reproducibility of existing methods is needed. Additionally, high-throughput could be a critical benefit for application in systematic epidemiologic studies, routine analysis and, in general, in cases in which the determination of these compounds in a high number of samples is required [21].

2. Experimental

2.1. Chemicals

Deionized water (18 mΩ cm) from a Millipore (Billerica, MA 01821, USA) Milli-Q water purification system was used to prepare all aqueous solutions. Lipoxygenase-derived hydroperoxides of arachidonic acid [*viz.* 15(S)-HpETE (15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid), 12(S)-HpETE (12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid), 5(S)-HpETE (5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid)], and linoleic acid [*viz.* 13(S)-HpODE (13S-hydroperoxy-9Z,11E-octadecadienoic acid), and 9(S)-HpODE (9S-hydroperoxy-10E, 12Z-octadecadienoic acid)] were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetic acid, formic acid, methanol, ethanol and acetonitrile from Scharlab (Barcelona, Spain), and ammonium formate and ammonium acetate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) were used for chromatographic separation. All chemicals were LC–MS grade and used without further purification.

2.2. Serum extraction from human individuals

Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ, USA) without additives (red top). The tube was not opened to ambient air and kept refrigerated until processing. Blood samples were processed within 1 h after collection and centrifuged at 4000 × *g* for 10 min to separate serum, which was placed in plastic tubes and stored at −80 °C until analysis. All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration [22], which were supervised by the ethical review board (ERB) of Reina Sofia Hospital (Córdoba, Spain) that approved the experiments. Individuals selected for this study were informed to obtain consent prior to this research.

2.3. Standard solutions and spiked serum samples

Stock standard solutions of all compounds were prepared in ethanol (500 μg/mL) and stored in amber vials at −80 °C under nitrogen atmosphere. Working solutions were prepared by dilution of the appropriate volume of stock ethanolic solution in serum pool aliquots to obtain spiked solutions. The spiked solutions were used for optimization of the sample preparation and chromatographic steps by coupling solid-phase extraction (SPE) and LC separation in an automated manner.

2.4. Automated sample treatment

On-line sample preparation was carried out with an automated SPE workstation Prospekt-2 system (Supplementary Fig. 1) from Spark Holland (Emmen, The Netherlands). The workstation was composed of an automatic cartridge exchanger (ACE) and a high-pressure syringe dispenser (HPD) for SPE solvent delivery. The automated system was coupled to a Midas autosampler furnished with a 500 μL sample-loop. Peek tube (0.25 mm i.d.) from VICI (Houston, USA) was used for all connections between the different valves. Full automation of the extraction step was controlled by Sparklink software version 2.10 from Spark Holland. An optimization kit of Hysphere SPE cartridges (10 mm × 2.0 mm) packed with different stationary phases from Spark Holland was tested. The stationary phases were CN-SE (silica-based cyanopropyl phase, particle size 7 μm), C2-SE (silica-based ethyl phase, particle size 7 μm), C8 EC-SE (end-capped silica-based octyl phase, particle size 10 μm), C18 EC (end-capped silica-based octadecyl phase, particle size 7 μm), Resin GP (polymeric polydivinylbenzene phase, particle size 5-15 μm), Resin SH (strong-hydrophobic modified polystyrene-divinylbenzene phase, particle size 20–50 μm) and MM Anion (strong basic mixed-mode anion). Chromatographic separation was performed in a Mediterranea Sea C18 analytical column (3 μm, 150 mm × 4.6 mm, Teknokroma, Barcelona, Spain) that was thermostated at 25 °C. In the optimized method, 800 μL of deionized water was added by the Midas autosampler to the sample vial containing 200 μL serum and mixed thoroughly for 2 min just before starting the automated extraction process. The final volume of each serum solution was 1 mL. Then, the optimum C2 cartridge was automatically solvated with 2 mL MeOH (at 5 mL/min), conditioned with 2 mL 20% methanol aqueous solution with 0.1% formic acid (at 5 mL/min) and equilibrated with 1 mL 20% methanol aqueous solution with 0.1% formic acid (at 0.3 mL/min). Subsequently,

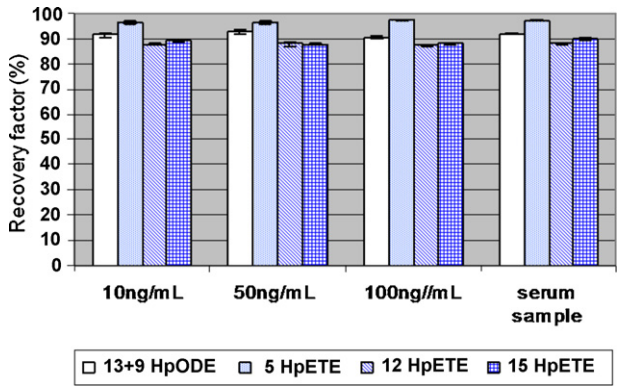

Fig. 1. Recovery factors (%) of SPE efficiency for each analyte. Human serum was spiked at different concentrations, showing that the breakthrough of the sorbent was not surpassed as the precision of the step was the same for all the assayed concentrations.

the sample was loaded into the cartridge (500 μL) by 1 mL 20% methanol aqueous solution with 0.1% formic acid (at 0.3 mL/min). Finally, elution of analytes was performed by forcing the LC mobile phase to pass through the SPE cartridge for 30 s. Then, the analytes in the eluate from the SPE cartridge were chromatographically separated in the analytical column prior to MS detection. The last step of solid-phase extraction was a purge tubing with 4 mL MeOH (at 5 mL/min) and 4 mL deionized water (at 5 mL/min) to prepare them for subsequent extraction steps. It was proven that cartridges can be reused up to 5 times without significant loss of extraction efficiency.

2.5. LC–MS/MS operating conditions

The SPE workstation system was on-line connected to an Agilent (Palo Alto, CA, USA) 1200 Series LC system, which consists of a binary pump, a thermostated column compartment and a vacuum degasser. Both the SPE and LC systems were configured for complete automation of analysis sequence. The chromatographic eluate was directly introduced in an Agilent 6410 triple quadrupole detector (QqQ) furnished with an Agilent Jet Stream Technology electrospray ion source. Nitrogen was provided by a high purity generator from CLAN Tecnológica (Sevilla, Spain) and used as the source gas, and nitrogen ultra pure (99.999%) from Carburos Metálicos (Sevilla, Spain) was used as collision gas. Agilent MassHunter Workstation was the software for data acquisition, qualitative and quantitative analysis. Separation of analytes was carried out in isocratic mode with 80:20 (v/v) methanol–water containing 0.02% formic acid mobile phase pumped at 1.0 mL/min.

The analytes were determined by ESI–MS/MS in negative mode by selected reaction monitoring (SRM). Triple Quadrupole MS and ionization chamber conditions were as follows: gas temperature, 335 °C; drying gas, nitrogen 10 L/min; nebulizer pressure, 43 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen 11 L/min; capillary voltage, 2900 V. A dwell time of 70 ms was applied to monitor all SRM transitions by virtue of the chromatographic separation of the target compounds. Both filter quadrupoles were adjusted at 0.7 mass units as full width at half maximum (FWHM) resolution. The data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis.

2.6. SRM-based quantitation of target analytes

Standard solutions were run by LC–MS/MS to build the corresponding calibration curve for each compound using the peak area as a function of the standard concentration of each compound. Stock standard solutions of 5-HpETE, 12-HpETE, 15-HpETE, 9-HpODE and 13-HpODE (500 μg/mL) were prepared in ethanol and stored in amber vials at −80 °C under nitrogen atmosphere. Calibration curves were constructed by spiking human serum with known amounts of standard solutions. The concentrations of target analytes at high level were 0.5 μg/mL. Ten calibration levels were prepared by 1:1 serial dilution in triplicate injection of three of them to set confidence levels. This calibration model was selected to correct matrix effects occurring during sample preparation (low recovery, saturation effects) and analysis (suppression).

3. Results and discussion

3.1. Optimization of LC–MS/MS analysis

Mass spectrometry optimization was initiated with a design aimed at finding the best ionization conditions for the target analytes. Ionization operating conditions were studied by direct injection of individual standard solutions using positive and negative ESI modes with different ionization agents and three levels of capillary voltage in the range 2600–3400 V. Negative ESI mode clearly showed a more efficient ionization by generation of dehydrated precursor ions $[M-H_3O]^-$ providing the best sensitivity for all hydroperoxides of eicosatetraenoic and octadecadienoic acids. The electrospray variables temperature, pressure, sheath gas temperature and nebulizer voltage were set with a multivariate response surface design in the ranges 165–350 °C, 3–60 psi, 280–380 °C and 1500–4000 V, respectively. An example of the surface responses obtained in this study is shown in Supplementary Fig. 2. Different ionization agents such as acetic acid and formic acid, and two volatile salts as ammonium acetate and ammonium formate were tested at different concentrations to compare the influence of pH on ionization. Formic acid led to the highest ionization efficiency by comparing signal-to-noise ratios and, therefore, to the best sensitivity by setting at the concentration 0.02% (v/v).

This study was followed by chromatographic tests under isocratic mode using acetonitrile–water mixtures at different ratios. Resolute separation with minimum chromatographic time was achieved by using an 80:20 (v/v) methanol–water solution excepting for both HpODE metabolites that were not resolved. Since both metabolites shared the same transitions from precursor to product ions, they were quantified together. With an isocratic protocol, equilibration of the chromatographic column is not required, thus increasing sample throughput.

Tandem mass spectrometry parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragmentations. The collision energy was changed from 5 to 60 eV after isolation of the target precursor ion for each analyte and the optimum values for all them are shown in Table 1. A unique transition was selected for each hydroperoxide with quantification purposes as secondary transitions were extremely low sensitive and they provided no extra levels of selectivity. Table 1 shows the target precursor and product ions selected for each prostanoid and optimum values found for filter voltage of the first quadrupole and collision energy. This study was completed by optimizing the influence of the dwell time in the range 50–250 ms, setting 70 ms for all transitions.

3.2. Optimization of SPE as sample preparation approach

Optimization of on-line sample preparation was aimed at obtaining maximum extraction efficiency and sample cleanup. The SPE workstation enabled to prepare automated sequences of analysis to test the influence of SPE sorbent, sample volume, loading solution, type of washing solvent and elution time by a univariate approach due to their discontinuous character. A pool serum

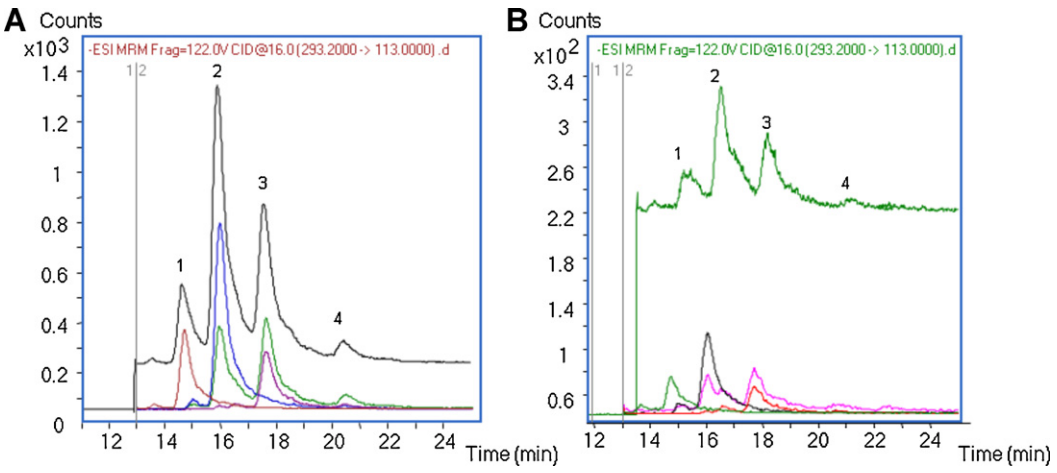

Fig. 2. Representative chromatograms of target eicosanoids in a serum sample spiked at 10 ng/mL (A) and a real serum sample (B). Analytes: (1) 9 + 13-HpODE (14.7 min); (2) 5-HpETE (15.9 min); (3) 12-HpETE (17.7 min); (4) 15-HpETE (20.5 min). The working conditions were those summarized in Section 2.

Table 1
Optimization of the MS/MS step for qualitative and quantitative determination of hydroperoxides of eicosatetraenoic and octadecadienoic acids.

Analyte	Precursor ion (*m*/*z*)	Voltage MS1 (V)	Product ion (*m*/*z*)	Collision energy (eV)	Quantitation transition
15-HpETE	317.2	115	273.2	8	317.2 → 273.2
12-HpETE	317.2	120	115.2	8	317.2 → 115.2
5-HpETE	317.2	120	113.2	12	317.2 → 113.2
13-HpODE	293.2	122	113.2	16	293.2 → 113.2
9-HpODE	293.2	122	113.2	16	293.2 → 113.2

spiked with target metabolites at 0.5 μg/mL was used for these tests. The suitability of different sorbent materials: polar and non-polar simple phases (CN, C2, C8 and C18), polymeric phases (resin general phase GP and strong hydrophobic SH) and a mixed-mode anionic phase were tested for optimum SPE retention/elution of the analytes into a single extraction method.

The optimization study was followed by independent tests for the three SPE steps: sample loading, washing and elution, which are directly related to analytes retention, interferences removal and recovery, respectively. Sample loading was optimized by testing different loading volumes and flow rates with a multivariate response surface design consisting of 16 experiments and 2 central points. The composition of loading solvent was set at 20% methanol aqueous solution with 0.1% formic acid. The use of an organic solvent and acid pH in this solution improved the retention of the target metabolites. However, methanol concentration should not exceed 20% to avoid protein precipitation in the workstation tubing. Acetonitrile as organic solvent for the loading step led to less precise results since protein precipitation was less controlled. Acidification of the sample ensured breaking of the proteins–target compounds binding; therefore, this fact did not affect the quantitation and recovery of the method.

The influence of the loading solvent volume was examined over the range 0.5–4 mL with flow rates ranging from 0.2 to 3 mL/min. As Table 2 shows, a volume of 1 mL was adopted since higher volumes caused partial elution of analytes and an optimum retention was obtained with slow flow rate: 0.3 mL/min. Retention of the target metabolites was improved by dilution of the sample by the loading solution. Sample dilution was associated with an increase of retention up to an 1:4 factor, which was used for further experiments.

The efficiency of a step for interferents removal was evaluated by circulating different volumes of washing solutions of different composition as Table 2 shows. Nevertheless, significant losses of metabolites were detected, even with aqueous solutions, which could be justified by the high polarity of the hydroperoxy functional group. For this reason, the washing step was omitted, and direct elution to the chromatographic column was performed by pumping the LC mobile phase – 80:20 (v/v) methanol–water – through

Table 2
Variables studied, range tested for each variable and optimum values selected in the optimization of SPE sample preparation for analysis of hydroperoxide metabolites.

Variable	Range tested	Optimum value
SPE sorbent	Hysphere CN, C2, C8 (EC), C18 HD, Resin GP and Resin SH	Hysphere C2
Sample dilution	1:1–1:5	1:4
Loading solvent		
Composition		
Organic proportion	From 0% to 50% of methanol and acetonitrile	20% methanol
Acidification	From 0% to 15% of formic acid and acetic acid	0.1% of formic acid
Volume	0.5–4 mL	1 mL
Flow rate	0.2–3 mL/min	0.3 mL/min
Washing solvent		
Composition		
Organic proportion	From 0% to 50% of methanol and acetonitrile	
Acidification	From 0.1% to 10% of formic acid	0 mL
Volume	0–3 mL	
Elution time	10–60 s	30 s

Table 3
Characteristics of the method.

Analyte	Equation	Linear range (ng/mL)	Coefficient of regression (R^2)	Limit of detection		Limit of quantification	
				(ng/mL)	On column (ng)	(ng/mL)	On column (ng)
15-HpETE	$y = -6.93 + 13.10C$	5–100	0.9999	1.50	0.15	5	0.5
12-HpETE	$y = -24.54 + 59.99C$	7–100	0.9975	2.10	0.21	7	0.7
5-HpETE	$y = -92.97 + 193.78C$	2.5–100	0.9971	0.75	0.08	2.5	0.25
9 and 13-HpETE	$y = -38.91 + 97C$	4–100	0.9973	1.20	0.12	4	0.4

the SPE sorbent. The elution step was governed by the time the LC mobile phase was pumped to the analytical column through the SPE system (*i.e.* elution time). Different elution times were studied by considering: (i) that the selected time should allow quantitative elution of analytes avoiding elution of interferents such as phospholipids that could produce ionization suppression in the electrospray of the mass analyzer and (ii) reducing as possible the excessive broadness of the chromatographic peaks. Thirty seconds was the selected time, after which the LC mobile phase was pumped directly to the analytical column without flowing through the SPE workstation. The recovery factor, defined as the proportion of analytes retained in the SPE cartridge, was evaluated by analysis of five replicates of non-spiked human serum samples by using a two-cartridge configuration. For this purpose, two cartridges were put in serial, so the sample was sequentially passed through them. In this way the amount of analyte no retained in the first cartridge could be retained in the second. Then, the compounds retained in both cartridges were sequentially eluted to the chromatographic column for independent analysis. Recovery was calculated as amount in cartridge 1/[amount in cartridge 1 + amount in cartridge 2], where the first and second cartridges were Hysphere C2 [23]. As Fig. 1 shows, recovery factors for all analytes were above 87.7%, which ensures quantitative retention in the cartridge and, therefore, validates internally the analytical method for determination of hydroperoxides of eicosatetraenoic and octadecadienoic acids.

3.3. Validation of the method

Validation of the method was supported on the deliberations of Food and Drug Administration (FDA) [24]. Validation of the analytical method included all the procedures to demonstrate that the method used for quantitative measurement of arachidonic and linoleic acid hydroperoxides in a given biological matrix such as serum is reliable and reproducible for the intended use. Fig. 2a and b shows two typical LC–MS/MS chromatograms and selected transitions corresponding to one of the serum samples of the target cohort and a spiked serum sample under the optimum conditions defined in the SOP reported here. Since spiked and non-spiked samples were used in this study, the endogenous levels of the target compounds have been implicitly established. The fundamental parameters for this validation include accuracy, sensitivity, precision, robustness and stability.

3.3.1. Accuracy study

The accuracy of the method was evaluated by analysis of five replicates of spiked human serum samples at three known levels in the range of expected concentrations (10, 50 and 100 ng/mL) by setting each variable at its optimum value. Spiked serum samples were analysed with the conventional single-cartridge configuration within the same day. The accuracy can be directly correlated with the factor estimated as follows: [final concentration – original concentration]/spiked concentration. The accuracy factor, as Fig. 1 shows, was above 86.4% for all target hydroperoxides. By comparison of spiked and non-spiked serum, the absence of ionization suppression effects can be confirmed with excellent precision. The fact that the recovery factor for the target compounds was lower than 100% (always higher than 86% and very closer to 100% in most cases) is due to the different chemical characteristics of the analytes, which are not equally retained by the sorbent. No breakthrough of the cartridge was surpassed despite the concentrations of the samples was within a high range as compared with the usual range of the analytes in human serum.

3.3.2. Evaluation of sensitivity and precision

A serum pool was used to run calibration plots for the target analytes using the peak area as a function of the standard concentration of each compound. Calibration curves were established by applying the standard addition method using stock-standard solutions and serum pool solutions. Analytical replicates ($n = 3$) of low, intermediate and high target hydroperoxides levels (10, 50 and 100 ng/mL, respectively), within the linear dynamic range, were injected to establish the confidence levels.

The limits of detection (LOD) and quantification (LOQ) for each analyte were calculated as the concentration that provided a signal three and ten times, respectively, higher than the noise background signal. The LODs ranged between 0.75 and 2.1 ng/mL (0.08–0.21 ng on-column), while LOQs were from 2.5 to 7.0 ng/mL (0.25–0.70 ng on-column). Table 3 shows the characteristics of the method.

Precision was evaluated with an experimental planning to estimate within-day variability, which was studied by analysis of four

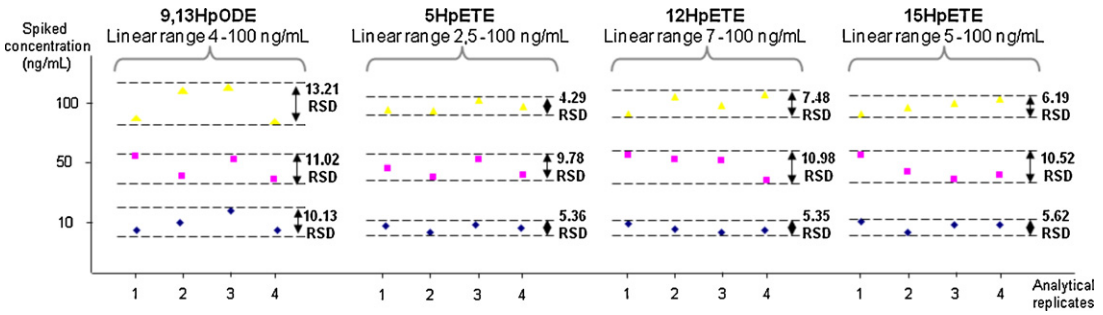

Fig. 3. Intra-day variability for each analyte, expressed as relative standard deviation (R.S.D.), by analysis of four replicates of spiked human serum samples at 3 known concentrations.

replicates of spiked human serum samples at three known concentrations (10, 50 and 100 ng/mL, respectively). The repeatability, expressed as relative standard deviation (R.S.D.), ranged between 4.3 and 13.2% for all target analytes. The results obtained are shown in Fig. 3 for the different hydroperoxides. As can be seen, the variability was maintained for the three concentration levels.

3.3.3. Robustness study

The robustness of the method was studied according to the number of analyses that can be carried out with the same cartridge without statistical effect on the accuracy of the method (95% confidence level). The SPE cartridge is the only factor to be modified in analysis by direct injection of human serum. One of the main advantages of SPE cartridges is their capability to be re-used without efficiency losses. Tests carried out with spiked serum samples ensured the re-usability of SPE cartridges for four analyses, which is highly appreciated taking into account the direct injection of serum in the SPE workstation without implementation of protein precipitation protocols that could enhance degradation kinetics of lipid hydroperoxides. Thus, a drastic reduction of the analysis costs is achieved, thus ensuring the robustness of the method with this reusability per cartridge.

3.4. Stability study

Due to the relevance of hydroperoxides stability on the final quality of the results, a detailed experimental planning was independently programmed to assess the stability of hydroperoxides under different conditions. The purpose was to determine the optimum conditions to complete the analysis without affecting the analytical properties of the method. The optimum conditions should be defined in the elaboration of the SOP report. Analyte stability in a biological fluid is a function of the storage conditions, the chemical properties of the analyte, the sample matrix, and sample container. In this study, spiked human serum samples stored in inert polypropylene Eppendorf-vials were used. Designed procedures were focused on the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (room temperature and refrigerate conditions) storage, and after freeze–thaw cycles.

3.4.1. Freeze–thaw stability

The stability of the analytes was determined after three freeze–thaw cycles. Two serum aliquots spiked at low concentrations (10 ng/mL) were stored at the freezer at −80 °C for 24 h and then, thawed at room temperature. When completely thawed, the samples were analysed and, subsequently refrozen for 24 h under the same conditions. The freeze–thaw cycle was repeated twice and, finally, the samples were analysed after the third cycle. The results obtained are shown in Fig. 4. As can be seen, there is a statistical loss of signal (95% confidence interval) for all hydroperoxides after the first thawing cycle. However, the loss of signal is attenuated for the second and third cycles. Therefore, it cannot be recommended to freeze–thaw serum samples in a clinical study.

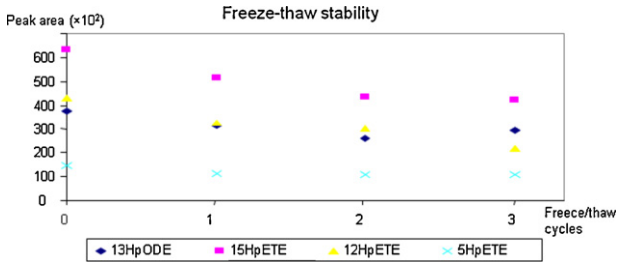

Fig. 4. Stability study of the target metabolites of a serum pool spiked at 10 ng/mL after 3 freeze–thaw cycles (ordinate axis: peak area).

3.4.2. Short-term temperature stability

A serum sample pool stored at −80 °C was thawed at room temperature and spiked with hydroperoxides at low concentration (10 ng/mL). Then, samples were analysed after 30, 60, 90 and 120 min, and then every hour up to 6 h. A similar study was also developed but working at 4 °C. In both cases, a decrease in the concentration of the target compounds was observed, as Fig. 5 shows. There was a significant difference in the MS signals for all hydroperoxides after 60 min at room temperature (decrease of 28.6% of the initial signal); while the analytes were stable at 4 °C for 3 h, except for 15HpETE, which was statistically affected after 60 min.

3.4.3. Long-term stability

Long-term stability was determined by analysis of aliquots of a spiked serum pool stored (10 ng/mL) at −80 °C. This study was developed for three weeks with analyses after 2, 5, 7, 9, 12, 14, 16 and 19 days. As can be seen, only the stability of 5HpETE can be statistically ensured after 12 days (95% confidence interval). The stability of the rest of compounds was significantly affected after 9 days, which should be set as the maximum storage time for serum samples with this SOP. The MS signals for all samples were compared to those obtained in the control analysis for the first day of long-term stability testing. The results obtained are shown in Fig. 6.

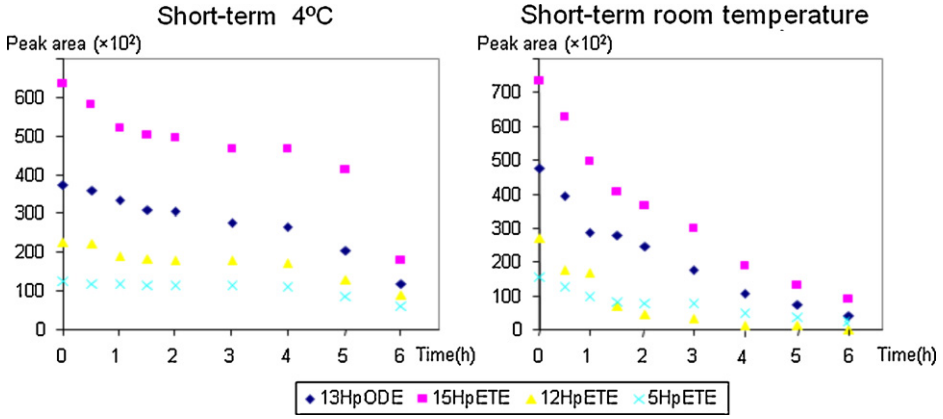

Fig. 5. Short-term stability study of the analytes of aliquots from a serum pool spiked at 10 ng/mL under two different conditions: 4 °C and room temperature (ordinate axis: peak area).

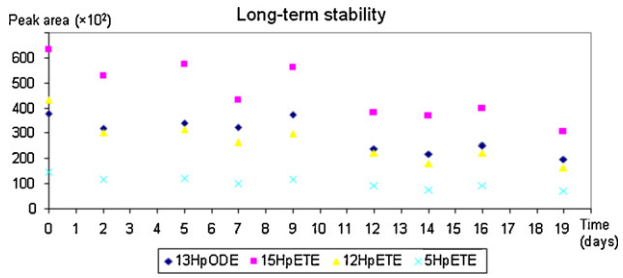

Fig. 6. Long-term stability study of the analytes of aliquots from a serum pool spiked at 10 ng/mL stored at −80 °C for 3 weeks.

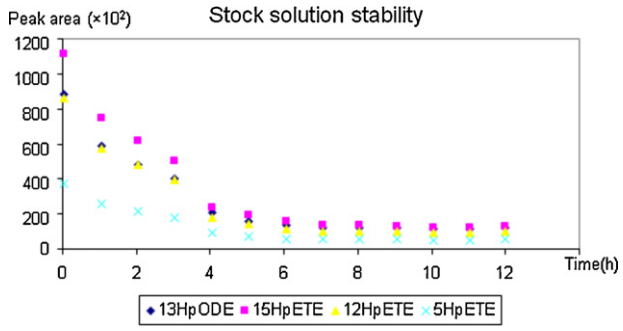

Fig. 7. Stability study of a 20 ng/mL stock solution of the target metabolites stored at room temperature and analysed every hour for 12 h.

3.4.4. *Stability of the stock solution*

A multistandard solution (20 ng/mL) was freshly prepared to be used as reference for this study. After preparation, the solution was analysed; stored at room temperature and analysed every hour for 12 h. The results, illustrated in Fig. 7, revealed that the stability of multistandard solutions is considerably affected at room temperature. In fact, significant differences (95% confidence interval) can be detected after 1 h at room temperature (decrease of 30.3% of the initial signal) justifying the relevance of this stability study.

4. Conclusions

A standard operation protocol for targeting analysis of hydroperoxy eicosatetraenoic and octadecadienoic acids in human serum has been developed by automated solid-phase extraction and liquid chromatography–mass spectrometry in selected reaction monitoring. A programmed validation study implemented robustness and stability tests for proper analysis of these metabolites of clinical relevance. After reception, the samples could be stored at −80 °C for 9 days with not statistically significant losses of the target lipid hydroperoxides.

The data obtained in this research suggest that preliminary steps should be considered in a metabolomics analysis by encompassing the different criteria for sample selection and storage and operations to be carried out prior to analysis. The relevance of these steps on the quality of the final results evidences the indispensable inclusion of such section in the elaboration of SOPs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.07.080.

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