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Integrated identification/confirmatory and targeted analysis of epoxyeicosatrienosic acids in human serum by LC–TOF MS and automated on-line SPE–LC–QqQ MS/MS

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ABSTRACT

A combined strategy is here proposed for qualitative/quantitative targeted analysis of epoxyeicosatrienoic acids (EETs) in human serum. Identification of EET regioisomers was initially carried out by LC–TOF MS in high accuracy mode under optimum conditions for chromatographic separation of the four isomers with an isocratic method using 40:40:20 (v/v/v) methanol–acetonitrile–water containing 0.02% acetic acid. Confirmatory analysis was supported on MS/MS experiments using the hybrid QqTOF mass analyzer by targeted fragmentation of the precursor ion fitting with the molecular formula $C_{20}H_{32}O_3$ (319.2279 m/z). Identification of selective fragment ions in high accuracy mode enabled the localization of the epoxy functional group and, therefore, the assignation of chromatographic peaks to each EET isomer. After qualitative analysis, an automated method was developed for analysis of EETs in human serum by direct analysis using an on-line platform based on SPE–LC–QqQ MS/MS in selected reaction monitoring. Recovery factors estimated with a dual-cartridge configuration were above 87% for all metabolites either using non-spiked and spiked serum at three different concentrations. Precision, calculated as within-laboratory repeatability and expressed as relative standard deviation, ranged from 2.5 to 9.9% with detection limits below 0.15 ng mL^{-1}. The optimization of the method was completed with a stability study under different conditions to assess the suited conditions for analysis of EET intermediate metabolites. Finally, concentration ranges of EETs were measured in nine healthy individuals.

1. Introduction

20-Carbon polyunsaturated fatty acids dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid (20:3$n-6$, 20:4$n-6$ and 20:5$n-3$, respectively) are the precursors of hormone-like eicosanoids, a wide variety of lipid mediators. Eicosanoids are formed through the action of a set of oxygenase enzymes such as cyclooxygenases, lipoxygenases and cytochrome P450 isozymes (CYP) [1,2]. The CYP class is divided into two groups: CYP epoxygenases, which catalyze formation of the epoxyeicosatrienoic acids (EETs), and the CYP *o*-oxidases, which catalyze hydroxylation of arachidonic acid at positions from ω16 to ω20 [3,4]. CYP epoxygenases produce four EET regioisomers from arachidonic acid: 5,6-, 8,9-, 11,12- and 14,15-EET. Each CYP epoxygenase catalyzes the formation of the four EET regioisomers indifferently, but one or two usually are the predominant products.

The primary function of EETs is to act as autocrine and paracrine mediators in the cardiovascular and renal systems. By modulating ion transport and gene expression, EETs trigger vasorelaxation and produce antiinflammatory and profibrinolytic effects [5]. EETs are potent vasodilators, especially in small arteries [6]. Also, they act on smooth muscle to open calcium-activated potassium channels, which results in membrane hyperpolarization and vasorelaxation [7–10]. Increased cerebral blood flow [11], protection of neurons [12] and astrocytes [13] from ischemic cell death by EETs have also been reported [14]. They also play a role in the regulation of angiogenesis and tumor growth [15].

Abbreviations: ACE, automatic cartridge exchanger; C18 EC, end-capped silica-based octadecyl phase; C18 HD, end-capped silica based phase with a high density of octadecyl chains; C2, silica-based ethyl phase; C8 EC, end-capped silica-based octyl phase; CN, silica-based cyanopropyl phase; CYP, cytochrome P450 isozymes; EETs, epoxyeicosatrienoic acids; FWHM, full width at half maximum; HPD, high-pressure syringe dispenser; JSESI, jet stream technology electrospray ion source; MM Anion, strong basic mixed-mode anion; Resin GP, polymeric polydivinylbenzene phase; Resin SH, strong-hydrophobic modified polystyrene-divinylbenzene phase; RSD, relative standard deviation

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Studies to determine the clinical importance of CYP arachidonic acid metabolites have created the need for sensitive, selective, and reproducible methods for measuring EET metabolites in human biological fluids. A number of methods have been developed for the detection and quantification of these metabolites: GC–MS, GC–MS/MS [16,17], capillary electrophoresis–UV [18], LC–fluorescence [19], radioimmunoassays [20], and enzyme immunoassays [21] have been used for quantitative analysis of these compounds in different tissues such as liver [22], human placenta [23], endothelial cells [24], and biofluids such as plasma [6] or urine [25]. GC–MS with chemical ionization has been the most commonly used approach for analysis of EETs by taking benefit of the selective information about the mass weight from each chromatographic signal. However, GC-based protocols seem not to be the most suited option for analysis of labile compounds such as EETs owing to tedious steps including TLC purification and derivatization required prior to analysis. Due to its combined sensitivity–selectivity, LC–fluorescence after derivatization seems to be a simple hyphenated approach [20], but the separation step is time-consuming, and the high background from the matrix largely interferes throughout the chromatogram, even after a solid-phase extraction clean-up step. Although immunoassay-based tests have been used for many different matrices, their limitations include high cost, low sensitivity, cross-reactivity, and long analysis time [26].

The most significant issue for quantification of these isomeric metabolites is selectivity when working with complex biological matrices. As a result, LC–MS and LC–MS/MS have been widely used as they are powerful analytical approaches that combine the resolving power of liquid chromatography with the detection selectivity of mass spectrometry. Several alternatives have so far been reported for the determination of EET such as LC–MS and LC–MS/MS. In general, these pose a number of disadvantages: either they do not exhibit the high selectivity necessary to work with complex matrices as serum, due to the fact that there exist a large number of compounds in these samples which have the same m/z as the interest compounds, which would make working in single ion monitoring (SIM) mode inadequate in terms of selectivity [27], or they require a prior derivatization step [28], or they are not fully automatic [29]. Similarly to other eicosanoids, the most important difficulty in analyzing EETs originates from rapid, extensive metabolism [30]. EETs degrade by several processes, including spontaneous hydration, conjugation to glutathione, and Ω or β oxidation [31].

The variability associated with sample storage or preliminary operations can be critical in targeted metabolomics approaches focused on a restricted set of metabolites that could be seriously affected [32]. It is known that unsuitable sample pretreatment protocols can lead to biased results owing to conversion or degradation of metabolites [33]. Increased interest exists in rapid handling of samples for metabolomics purposes, while turnover kinetics of some metabolites is known to be extremely fast. Accordingly, the time window between sampling and analysis has to be as short as possible; so, in this article we propose a fully-automated qualitative and quantitative methodology based on SPE–LC–MS/MS for the determination of EETs with previous confirmatory analysis by LC–QqTOF to ensure the separation–identification of the isomers under study.

2. Experimental

2.1. Chemicals

Deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system was used to prepare all aqueous solutions. (±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-EET), (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-EET), (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-EET) and (±)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-EET) 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 acetate from Sigma (Sigma-Aldrich, St. Louis, MO, USA) were used for the development of the analytical method. All chemicals were LC-grade and used without further purification, except for the use of the respective LC–MS grade solvents in LC–QqTOF analysis.

2.2. Serum samples 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 at 4 °C until processing. Blood samples were processed within 1 h after collection and centrifuged at $4000 \times g$ 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 of Helsinki [34], which were supervised by the ethical review board 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 (250 μg mL^{-1}) and stored in amber vials at −80 °C under nitrogen atmosphere. Working solutions or spiked samples were prepared by dilution of the appropriate volume of stock solutions in ethanol or in serum pool aliquots, respectively, then used for optimization of the chromatographic steps by LC–MS, identification of EET isomers by LC–QqTOF MS/MS and sample preparation by coupling solid-phase extraction SPE and LC–QqQ MS/MS in an automated manner.

2.4. LC–QqTOF MS/MS analysis

Identification and confirmation of the presence of epoxyeicosatrienoic acids were supported on LC–QqTOF MS/MS in negative ionization mode. The analytes were separated using an LC (series 1200, Agilent Technologies, Palo Alto, CA) equipped with a reversed-phase C18 Mediterranea Sea analytical column (Teknokroma, Barcelona, Spain) with 150 mm × 4.6 mm dimensions packed with 3 μm particle diameter. Column temperature was maintained at 20 °C. Separation of analytes was carried out in isocratic mode with 40:40:20 (v/v/v) methanol–acetonitrile–water containing 0.02% acetic acid, mobile phase pumped at 1.1 mL min^{-1} and 20 μL of the sample extracts were injected. The injector needle was washed for 12 times with 80% acetonitrile to avoid cross-contamination. Furthermore, the needle seat back was flushed for 15 s at a flow rate of 4 mL min^{-1} with 80% acetonitrile to clean it. The LC system was connected to a hybrid mass spectrometer formed by a dual quadrupole connected to a time-of-flight analyser (Agilent 6540 UHD Accurate-Mass QqTOF) equipped with an electrospray interface under the following operating parameters: capillary 3500 V, nebulizer 40 psi, drying gas 10 L min^{-1}, gas temperature 350 °C, filtering voltage 175 V, skimmer 65 V, Oct rf Vpp 750 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 60–1700 range. The instrument gave typical resolution 15000 FWHM (full width at half maximum) at m/z 112.9856 and 30,000 FWHM at m/z 1033.9881. To assure the desired mass accuracy of

recorded ions, a second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: 119.0362 (proton abstracted purine) and 1033.9881 *m*/*z* (protonated hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phospazine). Data were collected over the *m*/*z* 100–1700 range in centroid and profile modes at a rate of 1.0 spectrum per second in the extended dynamic range mode (2 GHz resolution).

2.5. Automated sample treatment

On-line sample preparation was carried out with an automated SPE workstation Prospekt-2 system (see Supporting information, Fig. 1) from Spark Holland (Emmen, The Netherlands), which includes 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.

Various Hysphere SPE cartridges from Spark Holland were initially tested for development of the SPE step, namely: CN (silica-based cyanopropyl phase, particle size 7 μm), C2 (silica-based ethyl phase, particle size 7 μm), C8 EC (end-capped silica-based octyl phase, particle size 10 μm), C18 EC (end-capped silica-based octadecyl phase, particle size 7 μm), C18 HD (end-capped silica based phase with a high density of octadecyl chains, 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). All cartridges were 10 mm × 2 mm i.d.

In the optimized procedure, a volume of 800 μL of a 0.2% acetic acid aqueous solution was added by the Midas autosampler to the sample vial containing 200 μL serum and mixed thoroughly for 2 min at 20 °C temperature just before starting the automated extraction process. The final volume of each serum solution was 1 mL. Thus, the optimum Resin GP cartridge was automatically solvated with 2 mL MeOH (at 5 mL min^{-1}), conditioned with 2 mL 0.2% acetic acid (at 5 mL min^{-1}) and equilibrated with 1 mL 0.2% acetic acid (at 0.5 mL min^{-1}). Subsequently, the sample was loaded into the cartridge (500 μL) by 2.2 mL 0.2% acetic acid (at 0.5 mL min^{-1}). Finally, elution of analytes was performed by forcing the LC mobile phase to pass through the SPE cartridge for 40 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 methanol (at 5 mL min^{-1}) and 4 mL deionized water (at 5 mL min^{-1}) to prepare the system for subsequent extraction steps. It was proven that cartridges can be reused for four times without statistical effect on precision and sensitivity; thus allowing a drastic reduction of the analysis costs taking into account that direct serum analysis is carried out without protein precipitation.

2.6. LC–QqQ MS/MS determination

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 6460 triple quadrupole detector (QqQ) furnished with an Agilent Jet Stream Technology electrospray ion source (JSESI). 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. The separation conditions were identical with those for the LC–QqTOF MS/MS analysis.

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^{-1}; nebulizer pressure, 34 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen 11 L min^{-1}; capillary voltage, 2600 V. A dwell time of 60 ms was applied to monitor all SRM transitions as this value provided the best 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.

2.7. SRM-based quantification 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 5,6-EET; 8,9-EET; 11,12-EET and 14,15-EET (250 μg mL^{-1}) were prepared in ethanol and stored in amber vials at −80 °C under nitrogen atmosphere. Calibration curves were constructed by spiking blank human serum pool with known amounts of standard solutions. The concentrations of target analytes at high level were 0.1 μg mL^{-1}. Ten calibration levels were prepared by 1:2 serial dilutions in triplicate injection of three of them to set confidence levels (0.02, 0.05 and 0.09 μg mL^{-1}). This calibration model was selected to correct matrix effects occurring during sample preparation (low recovery, saturation effects) and analysis (ionization/fragmentation suppression).

3. Results and discussion

3.1. Optimization of chromatographic separation

The study of the chromatographic separation was performed in negative ionization mode with acquisition in full scan by using one single quadrupole of the triple quadrupole mass analyzer. The parameters monitored for optimization were the height and width of chromatographic peaks to evaluate resolution and separation capability. Due to the non-polar character of the target compounds, a C18 analytical column was selected for chromatographic separation since this provided an optimum retention and resolution. The influence of ionization agent was studied by addition of different concentrations of acetic acid, formic acid and the corresponding ammonium formate and ammonium acetate salts. The best results in terms of peak area were obtained in isocratic mode with the mixture 40:40:20 (v/v/v) methanol–acetonitrile–water containing 0.02% acetic acid. With an isocratic protocol, equilibration of the chromatographic column is not required, thus increasing sample throughput, although from time to time is necessary a clean-up step of the analytical column. Flow rates between 0.5 and 1.5 mL min^{-1} and temperatures between 15 and 40 °C were also tested, finding optimum separation at 20 °C with 1.1 mL min^{-1} flow-rate.

3.2. Identification and confirmatory analysis of epoxyecosatrienoic acids in human serum by LC–QqTOF MS/MS

The correct identification of target compounds represents a critical prerequisite for analysis of isomer compounds. For this purpose, a combined analysis between MS and MS/MS was

planned to take benefit of mass accuracy for precursor ion and the resulting product ions generated in the collision cell. In this step, non-spiked and spiked serum samples (60 ng mL^{-1}) were treated with ethanol for deproteinization, which is one of the most common solvents used for this aim [35]. The volume of serum employed in this case was 100 μL, to which 200 μL of ethanol was added and mixed thoroughly for 2 min. Then, the precipitate was removed by centrifugation at 4 °C for 10 min at 20000*g*, and 10 μL of supernatant was injected in the system LC–TOF MS to localize chromatographic signals corresponding to the different isomers. Accurate mass spectrum were acquired in the range 250–400 *m*/*z*. Fig. 1 shows the extracted ion chromatogram obtained from a spiked serum sample (60 ng mL^{-1} of each EET) for the 319.2279 *m*/*z* (*m*/*z* window expressed as 10 ppm) that fits the molecular formula of the EET isomers. As can be seen several chromatographic peaks were detected for this *m*/*z* ratio. The accuracy error for these signals (a total of nine signals) ranged from 0 to 6.58 ppm. Isomers assignation was only possible by a targeted MS/MS analysis using the QqTOF hybrid analyzer after chromatographic separation by filtering the precursor ion at 319.2279 *m*/*z* in the first quadrupole and subsequent fragmentation with different collision energy values. The collision energy range studied was from 5 to 20 eV. Fragmentation of precursor ions with increasing collision energy did not provide improvement of the MS/MS spectra for target EET isomers. The spectra obtained at collision energies from 10 to 20 eV were characterized by a significant background resulting in complex but not selective MS/MS spectra. Nevertheless, at 5 eV as collision energy representative fragments of the target molecules were identified revealing the identity of the EET isomers by localization of the epoxy functional group (see Fig. 2). As can be seen, two different fragmentation schemes were obtained for EET isomers. The 14,15-EET and 8,9-EET experienced MS/MS rupture by the epoxy functional group, while 11,12-EET and 5,6-EET were fragmented by the annex methylene groups.

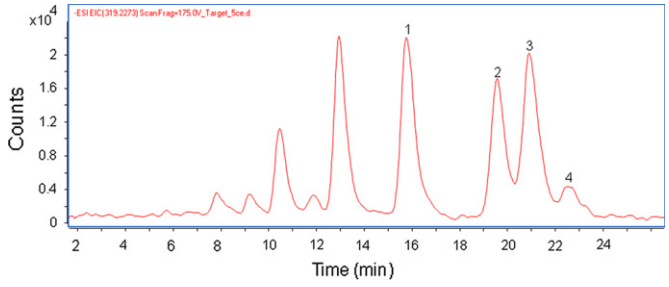

Fig. 1. Extracted ion chromatogram for the ion 319.2279 *m*/*z* that fits with the molecular formula of the EET isomers ($C_{20}H_{32}O_3$) generated by LC–TOF MS in high accuracy mode. Analytes: (1) 14,15-EET (15.8 min); (2) 11,12-EET (19.5 min); (3) 8,9-EET (20.9 min); (4) 5,6-EET (22.5 min).

3.3. Optimization of LC–MS/MS determination by SRM with a triple quadrupole mass analyzer

Mass spectrometry optimization started 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

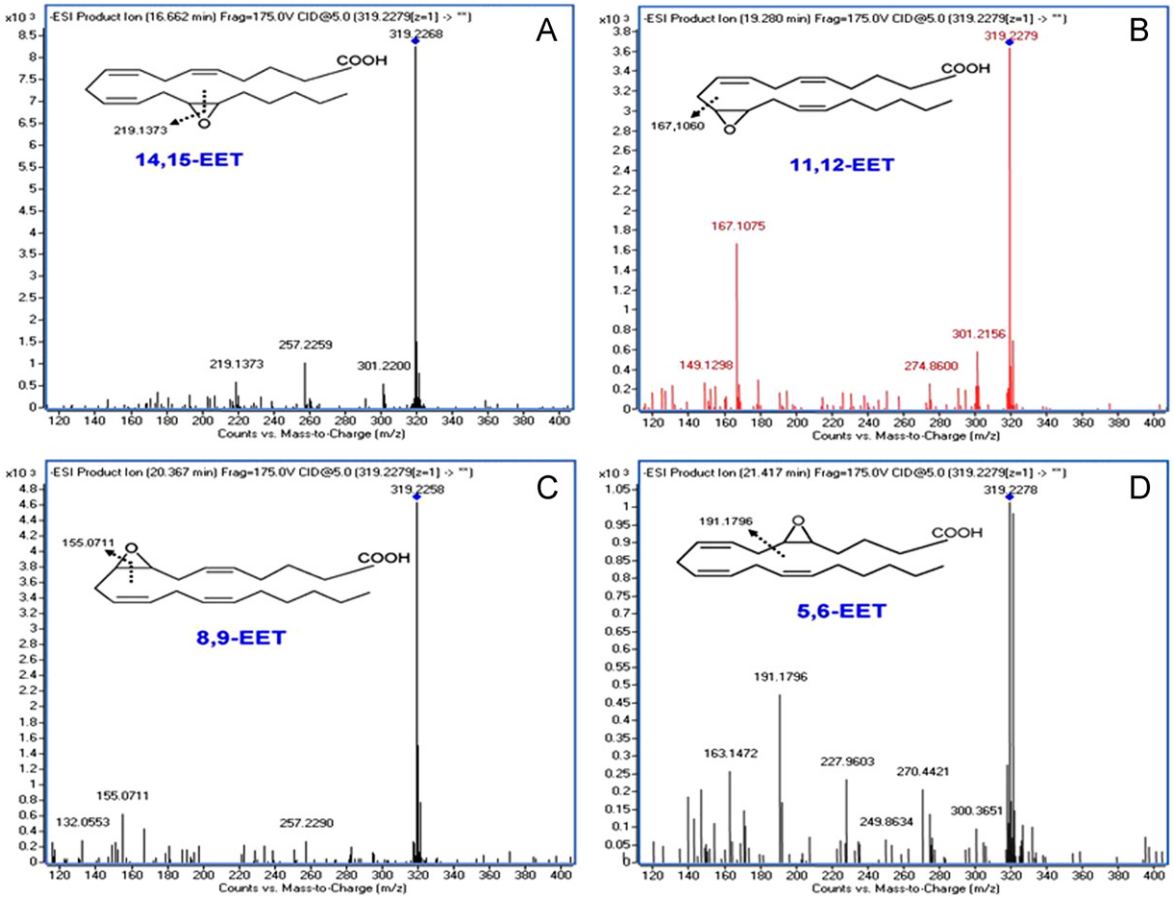

Fig. 2. Fragmentation spectra in negative ionization mode corresponding to the ion 319.2279 *m*/*z* for the target EETs. (A) 14,15-EET, (B) 11,22-EET, (C) 8,9-EET, and (D) 5,6-EET.

capillary voltage in the range 2700–4500 V by two electrospray ion (ESI) sources, one of which focalizes the spray at the entrance of the spectrometer with the aid of a N_2 current (JSESI). Negative mode with JSESI clearly showed a more efficient ionization by generation of $[M–H]^-$ precursors providing the best sensitivity for target epoxyeicosatrienoic acids. The electrospray variables temperature, pressure, sheath gas temperature, and capillary voltage were set with a multivariate response surface design in the ranges 165–335 °C, 23–60 psi, 280–380 °C and 2700–4500 V, respectively. 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. Acetic acid led to the highest ionization efficiency by comparing signal-to-noise ratios and, therefore, to the best sensitivity by setting the concentration at 0.02% (v/v).

Tandem mass spectrometry parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragmentations. The collision energy was varied from 0 to 20 eV after isolation of the target precursor ion for each analyte. The optimum collision energy for all analytes was 4 eV. A single transition was selected for each epoxyeicosatrienoic acid with quantification purposes due to the fact that secondary transitions were extremely low-sensitive. Table 1 shows the target precursor and product ions selected for each epoxyeicosatrienoic acid and the optimum values found for filtering 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 60 ms for all transitions.

3.4. Optimization of SPE as sample preparation approach

The different variables related to automated sample preparation were optimized by using a univariate approach to assess clean-up and retention–elution capabilities. 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 spiked with target metabolites at 0.1 μg mL^{-1} was used for these tests. The suitability of various sorbent materials including CN, C8 EC, C18 EC, C18 HD, Resin GP, Resin SH and MM anion cartridges for SPE was assessed. Resin GP cartridges were adopted for further testing as they provided the best overall recoveries in these preliminary tests, which were supported on the characteristics of this highly-hydrophobic material with mixed mechanisms based on polydivinyl–benzene interactions. Acidification of samples was also tested with a view to improving retention of EET isomers. Serum samples were thus spiked at a 0.1 μg mL^{-1} concentration and acidified with acetic acid before on-line SPE. Potential precipitation by effect of sample standing at pH acid on the autosampler during the whole analysis sequence was avoided by in situ acidification. To this end, variable volumes and concentrations of formic acid and acetic acid were added by the autosampler to the serum samples immediately before automated SPE was started. The most reproducible results were obtained by adding 800 μL of 0.2% acetic acid to 200 μL serum. Thus, SPE was efficiently conducted by in situ acidification.

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, interferents removal and recovery, respectively. Sample loading was optimized by testing loading volumes between 1 and 4 mL and flow rates between 0.3 and 3 mL min^{-1}. The loading solvent was pure water with 0.2% acetic acid. The presence of an organic solvent such as acetonitrile or methanol in this solution decreased the retention of the target metabolites. As Table 2 shows, a volume of 2.2 mL was adopted since higher volumes caused partial elution of analytes and an optimum retention was obtained with slow flow rate: 0.5 mL min^{-1} to favor interactions between EET isomers and cartridge particles.

The efficiency of a clean-up step was evaluated by circulating different volumes of washing solutions of different composition as Table 2 shows. Significant losses of metabolites were detected, even with aqueous solutions, which could be justified by the high polarity of the epoxy 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 – 40:40:20 (v/v/v) methanol–acetonitrile–water containing 0.02% acetic acid – through 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). This interval should allow quantitative recovery of the analytes while avoiding elution of interferences potentially causing ionization suppression in the mass analyzer electrospray and excessively broad chromatographic peaks. In this sense, the elution of the analytes was ensured after 40 s, after which the LC mobile phase was pumped directly to the analytical column by passing the SPE workstation.

Table 1
Optimization of the MS/MS step for quantitative analysis of epoxyeicosatrienoic acids by SRM.

Analyte	Precursor ion (*m/z*)	Voltage MS1 (V)	Product ion (*m/z*)	Collision energy (eV)	Quantitation transition
14,15-EET	319.2	120	219.2	4	319.2→219.2
11,12–EET	319.2	82	167.0	4	319.2→167.0
8,9–EET	319.2	99	154.9	4	319.2→154.9
5,6–EET	319.2	94	191.0	4	319.2→191.0

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

Variable		Range tested		Optimum value
SPE sorbent		Hysphere CN, C8 (EC), C18 EC, C18 HD, Resin GP, Resin SH and MM anion		Resin GP
Sample dilution		1:1–1:7		1:5
Loading solvent	Composition (%)	Organic proportion	From 0 to 30 of methanol and acetonitrile	Water with 0.2 of acetic acid
		Acidification	From 0 to 2,5 of formic acid and acetic acid	
	Volume (mL)	1–4		2.2
	Flow rate (mL min^{-1})	0.3–3		0.5
Washing solvent	Composition (%)	Organic proportion	From 0 to 30 of methanol and acetonitrile	0
		Acidification	From 0 to 10 of acetic acid	
	Volume (mL)	0–3		0
Elution time (s)		10–60		40

3.5. Validation of the method

The accuracies of the method and potential matrix effects were studied by analysis of non-spiked and spiked human serum samples by setting each variable at its optimum value. First, the recovery factor, defined as the proportion of analytes retained in the SPE cartridge, was evaluated by analysis of four 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 detected after elution from cartridge 1/[amount detected after elution from cartridge 1+amount detected after elution from cartridge 2], where the first and second cartridges were Hysphere Resin GP [36]. As Table 3 lists, recovery factors for all analytes were above 87.0%, which ensures quantitative retention in the cartridge and, therefore, validates internally the analytical method for determination of epoxyeicosatrienoic acids.

Four replicates of spiked human serum samples at three known levels in the range of expected concentrations (5, 12.5 and 25 ng mL^{-1}) were analyzed with the single configuration within the same day. In this case, the accuracy can be directly correlated with the factor estimated as follows: [final concentration–original concentration]/spiked concentration. In this case, apparent recoveries were above 86.9% for all epoxyeicosatrienoic acids, which ensures minimum ionization suppression effects with excellent precision. The results thus obtained are listed in Table 3.

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 epoxyeicosatrienoic acid levels (2.5, 5 and 25 ng mL^{-1}, respectively), within the linear dynamic range, were injected to establish the confidence levels.

By using a serum pool, the limit of detection (LOD) and that of quantification (LOQ) of the method 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 of the method ranged between 0.02 and 0.03 ng, while LOQs were from 0.06 and 0.10 ng on-column. Table 4 shows the characteristics of the method.

Within-laboratory repeatability was evaluated in a single experimental set-up with five replicates of spiked human serum samples at three known concentrations (2.5, 5 and 25 ng mL^{-1}). The results obtained are listed in Table 3. The repeatability, expressed as relative standard deviation (RSD), ranged between 2.5 and 9.9% for all target analytes.

3.6. Stability study and application to human serum samples

Due to the relevance of epoxyeicosatrienoic acids stability on the final quality of results, a study was programmed to assess the

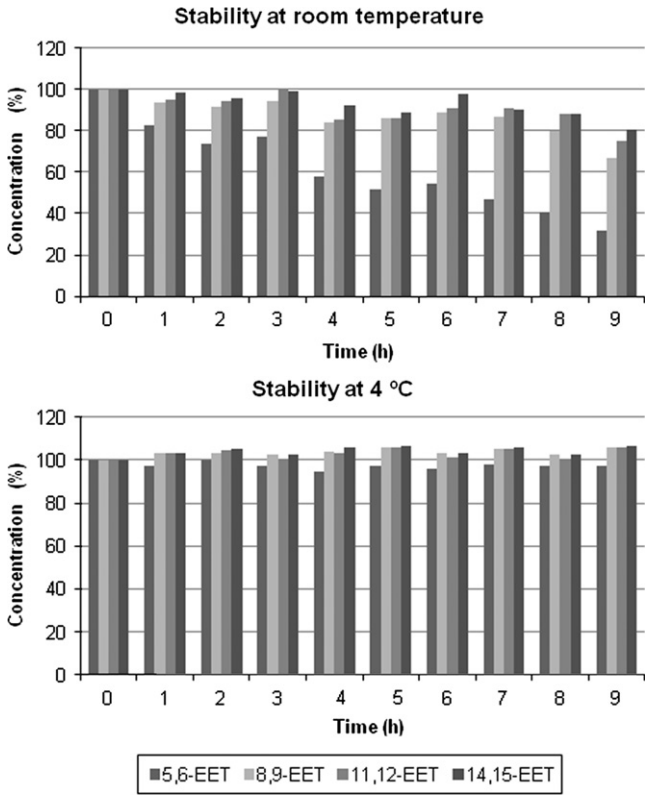

Fig. 3. Stability study of EETs isomers spiked to a serum pool at concentration of 9 ng mL^{-1}. The serum pool was thawed at room temperature (A) and at 4 °C (B) with subsequent analysis at a frequency of 1 aliquot h^{-1} for 9 h.

Table 3
Whitin-laboratory repeatability expressed as relative standard deviation (%) and recovery factor (%) estimated for each analyte with two configurations: (1) two-cartridge configuration, (2) one-cartridge configuration.

	Concentration (ng mL^{-1})	Analyte			
		14,15-EET	11,12-EET	8,9-EET	5,6-EET
Repeatability (%)	2.5	9.9	5.9	6.8	4.4
	5	8.4	5.9	6.0	5.6
	25	2.5	2.5	3.0	3.6
Recovery (%) **(1)**	0	96.0	95.4	93.0	87.0
Recovery (%) **(2)**	5	96.8	96.5	95.5	88.7
	12.5	98.0	98.0	96.6	92.4
	25	95.9	95.4	93.1	86.9

[a]Expressed as percent of amount in cartridge 1/[amount in cartridge 1+amount in cartridge 2].
[b]Expressed as percent of [final concentration–initial concentration]/added concentration.

Table 4
Characteristics of the method.

Analyte	Retention time (min)	Linear range (ng mL^{-1})	Coefficient of regression (R^2)	Limit of detection		Limit of quantification	
				(ng mL^{-1})	On column (pg)	(ng mL^{-1})	On column (pg)
14,15-EET	16.40	0.30–75	0.9981	0.09	9	0.30	30
11,12-EET	19.40	0.40–75	0.9969	0.12	12	0.40	40
8,9-EET	20.20	0.50–75	0.9983	0.15	15	0.50	50
5,6-EET	21.20	0.50–75	0.9941	0.15	15	0.50	50

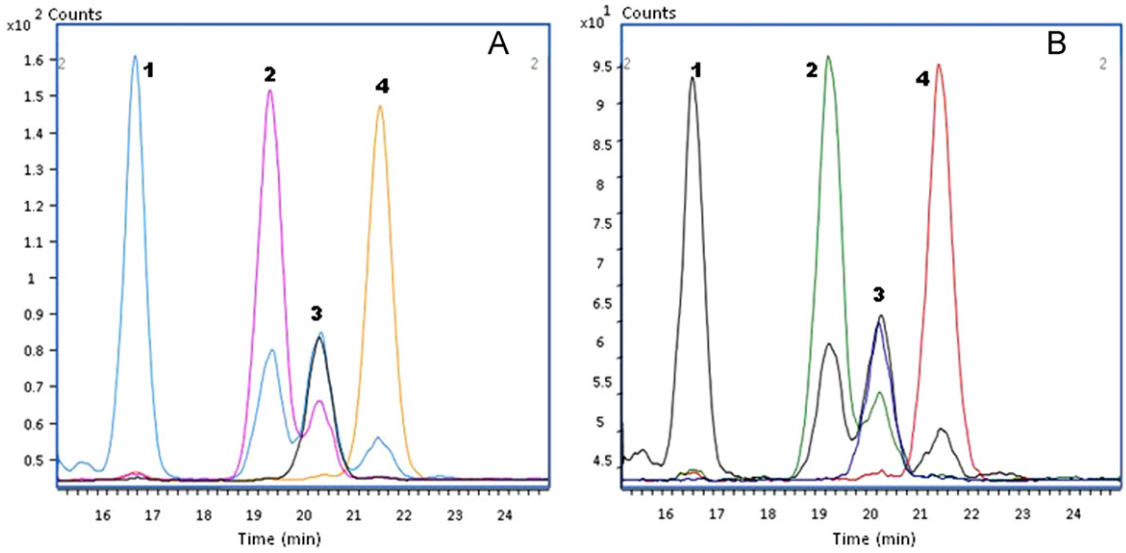

Fig. 4. Representative SRM chromatograms of EET isomers in a serum sample spiked with 0.5 ng mL^{-1} of EETs (A), and in a non spiked serum sample (B), generated by SPE–LC–QqQ MS/MS. Analytes: 1, 14,15-EET (16.4 min); 2, 11,12-EET (19.4 min); 3, 8,9-EET (20.2 min); 4, 5,6-EET (21.6 min).

stability of epoxides under optimal operation conditions. A serum sample pool stored at −80 °C was thawed at room temperature and spiked with epoxyeicosatrienoic acids at medium concentration (9 ng mL^{-1}). Then, samples were analyzed each hour up to 9 h. A similar study was also developed but working at 4 °C. In both cases, a decrease in the concentration of the target compounds under study was observed, as Fig. 3 shows. There was a significant difference in the MS signals for all epoxyeicosatrienoic acids after 9 h at room temperature (decrease at least 19.2% of the initial signal). Special mention deserves 5,6-EET, which was statistically affected after 5 h at room temperature, with a decrease of almost 50% of the initial signal. On the other hand, EETs were stable at 4 °C for at least 9 h, which ensured their quantitative determination after blood extraction.

In order to demonstrate the applicability of the proposed method, this was applied to determine the target analytes in serum from 9 apparently healthy volunteers. The 11,12-EET was found in all samples analyzed in a concentration range from 0.90 to 1.96 ng mL^{-1}. However, the other target compounds (*i.e.*, 5,6-EET, 8,9-EET and, 14,15-EET) were only found in 4 of the 9 samples tested. The concentration ranged from 0.88–1.13, 0.60–2.63 and, 0.68–2.17 ng mL^{-1} for 5,6-EET, 8,9-EET and, 14,15-EET, respectively. Fig. 4A and B shows two typical LC–MS/MS chromatograms and selected transitions corresponding to a spiked serum sample with 0.5 ng mL^{-1} of EETs and one of the serum samples of the target cohort under the optimum conditions defined in this research.

4. Conclusions

A combined strategy has been developed for analysis of EET isomers in human serum. The proposed method has been designed to process serum samples for qualitative and quantitative determination of metabolites produced in the CYP epoxygenase pathway. Identification and confirmatory analysis of epoxyecosatrienoic acids in human serum has been carried out by LC–QqTOF (a combined analysis between MS and MS/MS) in high accuracy mode for precursor and product ions. Quantitative analysis has been supported on a SRM method using a triple quadrupole mass analyser. An exhaustive stability study was also carried out in order to elucidate the correct analysis conditions. The resulting method leads to minimization of human intervention, high precision and high throughput, key characteristics for routine analysis in epidemiological or clinical studies. The overall method has been properly validated by application to human serum samples.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.018.

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