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# Confirmatory and quantitative analysis of fatty acid esters of hydroxy fatty acids in serum by solid phase extraction coupled to liquid chromatography tandem mass spectrometry

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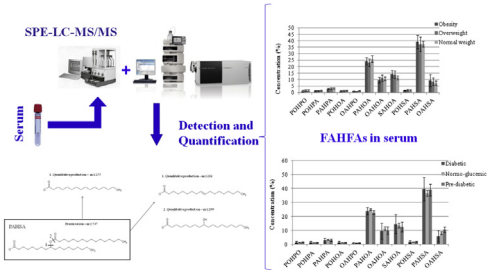
## HIGHLIGHTS

- Automated method to determine fatty acid esters of hydroxy fatty acids (FAHFAs).
- The method has been optimized to maximize sensitivity.
- The method was based on the on-line coupling between SPE and LC–MS/MS.
- The method was used to determine FAHFAs in a cohort of serum samples from volunteers.

## GRAPHICAL ABSTRACT

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## ABSTRACT

A novel class of endogenous mammalian lipids endowed with antidiabetic and anti-inflammatory properties has been recently discovered. These are fatty acid esters of hydroxy fatty acids (FAHFAs) formed by condensation between a hydroxy fatty acid and a fatty acid. FAHFAs are present in human serum and tissues at low nanomolar concentrations. Therefore, high sensitivity and selectivity profiling analysis of these compounds in clinical samples is demanded. An automated qualitative and quantitative method based on on-line coupling between solid phase extraction and liquid chromatography–tandem mass spectrometry has been here developed for determination of FAHFAs in serum with the required sensitivity and selectivity. Matrix effects were evaluated by preparation of calibration models in serum and methanol. Recovery factors ranged between 73.8 and 100% in serum. The within-day variability ranged from 7.1 to 13.8%, and the between-days variability varied from 9.3 to 21.6%, which are quite acceptable values taking into account the low concentration levels at which the target analytes are found. The method has been applied to a cohort of human serum samples to estimate the concentrations profiles as a function of the glycaemic state and obesity. Statistical analysis revealed three FAHFAs with levels significantly different depending on the glycaemic state or the body mass index. This automated method could be implemented in high-throughput analysis with minimum user assistance.



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**Abbreviations**

| | |
|---|---|
| ACE | automatic cartridge exchanger |
| BMI | body mass index |
| FAHFAs | fatty acid esters of hydroxy fatty acids |
| FWHM | full width at half máximum |
| HPD | high-pressure syringe dispenser |
| NL | Neutral Loss scan mode |
| OA | oleate |
| OAHOA | oleic acid-hydroxy-oleic acid |
| OAHSA | oleic acid-hydroxy-stearic acid |
| 12-OAHSA | oleic acid-12-hydroxy-stearic acid |
| PAHOA | palmitic acid-hydroxy-oleic acid |
| PAHPA | palmitic acid-hydroxy-palmitic acid |
| PAHSA | palmitic acid hydroxystearic acid |
| 12-PAHSA | palmitic acid-12-hydroxy-stearic acid |
| PO | palmitoleate |
| POHPA | palmitoleic acid-hydroxy-palmitic acid |
| POHPO | palmitoleic acid-hydroxy-palmitoleic acid |
| 12-POHSA | palmitoleic acid-12-hydroxy-stearic acid |
| SA | stearate |
| 12-SAHSA | stearic acid-12-hydroxy-stearic acid |
| SAHOA | stearic acid-hydroxy-oleic acid |
| SPE | solid-phase extraction |
| SRM | selected reaction monitoring |
| T2DM | type-2 diabetes mellitus |

## 1. Introduction

In the last decades the prevalence of metabolic syndrome has increased in developed countries, mainly because of the rise of obesity [1]. Metabolic syndrome is a collection of risk factors associated to a higher risk for cardiac diseases, atherosclerosis, fatty liver disease and diabetes [1–3]. These diseases share risk factors, being the most important obesity and insulin resistance [1]. Nowadays, the most common disease in patients affected by metabolic syndrome is diabetes mellitus. In fact, type-2 diabetes mellitus (T2DM) is increasing worldwide at an epidemic rate, which is expected to reach 592 million inflicted individuals by 2035 as compared to 382 million reached in 2013 [1,4]. Obesity is one of the major risk factors for T2DM, since around 85% of subjects with T2DM are overweighted or obese [5]. Thus, the body mass index (BMI) is not only associated with chronic low-grade inflammation and increased oxidative stress, but also with insulin resistance and metabolic dysregulation [2,5].

Recently, a novel class of endogenous mammalian lipids endowed with antidiabetic and anti-inflammatory properties has been found [6–11]. Yore et al. in 2014 referred to this class of natural-occurring lipids as fatty acid-hydroxy fatty acids, abbreviated as FAHFAs [6,7]. The authors identified 16 FAHFAs by different combinations of the main long-chain fatty acids (palmitate, oleate, stearate or palmitoleate) conjugated to a hydroxylated version of one of the same set of fatty acids [7]. In that research, the FAHFAs content of blood, serum and adipose tissue taken from diabetic mice were compared to that from normal mice. Yore et al. reported that 6 of the 16 FAHFA species were upregulated by GLUT4 overexpression, the unique insulin-sensitive glucose transporter protein [7]. The most dramatically upregulated FAHFA in the overexpressing GLUT4 was palmitic acid hydroxystearic acid (PAHSA) [7]. In humans, the authors observed a strong association between PAHSA and insulin sensitivity, being PAHSA levels significantly lower in insulin-resistant individuals as compared to insulin-sensitive cases [6,7]. Additionally, the characterization of the most common FAHFAs found in normal tissues and serum [6] revealed that their levels were similar to other signalling lipids such as prostacyclins, prostaglandins, steroids and endocannabinoids. Research on FAHFAs has revealed multiple effects that improve glucose-insulin homeostasis, which suggests that restoring PAHSA levels in insulin-resistant individuals could have beneficial metabolic effects. In fact, Yore et al. showed that administration of these fatty acids to mice improved glucose uptake from blood, enhanced insulin secretion and relieved obesity-associated inflammation, suggesting that these naturally occurring fats could be used for diabetes therapy [6].

Despite the potential of these novel lipids for treatments of diabetes [6,9,10], further studies are needed both to establish their normal physiological levels in humans and to study their evolution after a specific treatment. However, the low nanomolar concentrations at which FAHFAs are present in serum make necessary the development of a method with enough sensitivity to determine FAHFAs, even in deficiency state. One option is to preconcentrate FAHFAs by the implementation of a solid-phase extraction (SPE) step, which has been used for quantitation of other compounds found at similar concentrations to FAHFAs such as prostaglandins [10], prostanoids [11,12], steroids [13,14], eicosanoids [15,16] or endocannabinoids [17,18]. In this research an automated qualitative and quantitative method based on on-line coupling of SPE and liquid chromatography–tandem mass spectrometry (LC–MS/MS) to maximize sensitivity has been developed for determination of FAHFAs. The method has been further applied to a cohort of individuals to evaluate the influence of the glycaemic state on FAHFA levels.

## 2. Materials and methods

### 2.1. Reagents

Palmitic acid-12-hydroxy-stearic acid (12-PAHSA), palmitoleic acid-12-hydroxy-stearic acid (12-POHSA), stearic acid-12-hydroxy-stearic acid (12-SAHSA) and oleic acid-12-hydroxy-stearic acid (12-OAHSA) were purchased from Cayman Chemicals (Ann Arbor, MI, USA) in solution. Concretely, standard solutions contained 1 mg of each FAHFA in 200 μL of methyl acetate (5000 mg $L^{-1}$). Working solutions of each standard were prepared diluting the commercial standards at 500 μg $L^{-1}$ in chromatographic grade methyl acetate from Scharlab (Barcelona, Spain).

Chromatographic grade methanol and ammonium hydroxide were purchased from Scharlab (Barcelona, Spain), while mass spectrometry grade ammonium acetate was purchased from Fluka (Spain). Deionized water from a Millipore Milli-Q water purification system was used for preparation of all aqueous solutions.

### 2.2. Instruments and apparatus

A microcentrifuge Sorvall Legend Micro 21R from Thermo Scientific (Waltham, MA, US) was used to separate the phases after protein precipitation. On-line sample preparation was carried out with an automated SPE workstation Prospekt-2 system from Spark Holland (Emmen, The Netherlands), which included an automatic cartridge exchanger (ACE) and a high-pressure syringe dispenser (HPD) for solvent delivery. The automated system was coupled to a