Application of Solid Phase Microextraction for Quantitation of Polyunsaturated Fatty Acids in Biological Fluids

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SUPPORTING INFORMATION

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Summary

This supporting information file includes additional results and information as described in the text of the main article including detailed experimental protocol as well as supporting results and discussions in the following topics: 1) chemical and reagents information, 2) LC-ESI-MS typical chromatogram of fatty acids under study; 3) Determination of Matrix Effect and Ionization Suppression; 4) SPME method development; 5) Extraction efficiency of the SPME method in PBS; and 6) Evaluation of matrix effect using Sample Extract Dilution Method

Detailed Experimental Protocol

1.1.   Chemicals and Reagents

Methanol, acetonitrile, 2-propanol (all HPLC grade) were purchased from Caledon Labs (Georgetown, ON). LC-MS grade formic acid was obtained from Fisher Scientific (Ottawa, ON). Biocompatible SPME C18 probes (C18, 45 µm thickness, 15 mm coating length) were provided by Supelco (Bellefonte, PA). Human serum Albumin, essentially fatty acid free was obtained from Fluka (Sigma-Aldrich Oakville, ON). Fatty acids were selected based on (i) their hydrocarbon chain length and (ii) number and position of double bonds. Docosahexaenoic acid (DHA), Docosapentaenoic acid (DPA), Adrenic acid, Docosatrienoic acid (DTA), Eicosapentaenoic acid (EPA), Arachidonic acid, Eicosatrienoic acid (ETA), Stearidonic and α-Linolenic acid were purchased from Cayman Chemical (Ann Arbor, MI) and stored at −20°C. Individual stock solutions containing 1 mg/mL of each standard were prepared by dissolving the analytes in HPLC-grade methanol. For instrument calibration, working standard solutions with known concentrations of standard fatty acids were prepared by mixing adequate volumes of diluted stock solutions and adding acetonitrile as needed. All stocks and working standards were stored at -20°C. A phosphate-buffered saline (PBS) solution was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.24 g of potassium phosphate monobasic, and 1.44 g of sodium phosphate dibasic in 1 L of purified water (pH = 7.4). Extraction standards also were prepared daily by dilution to 1 µg/mL with PBS buffer solutions at pH 7.4 to mimic the physiological conditions, while keeping the organic solvent content of all extraction standards at ≤1% (v/v). PBS buffer solutions have been used for this study to mimic the physiological conditions for the initial SPME method optimization as a matrix-free environment.

1.2.   Determination of Matrix Effect and Ionization Suppression

Measurements of matrix effect were performed by using the post-extraction spiked method [1] in triplicates (n = 3). The neat solvent used in this case was the desorption solution (acetonitrile), which was spiked at a medium concentration level (50 ng/mL). The extracts from plasma samples were spiked with fatty acid standards at the same concentration as the neat solvent, and samples were individually injected into the LC/MS/MS system for quantification. In addition, matrix effect was evaluated using "sample extract dilution". In this technique, the final sample

extract from spiked human plasma is diluted by different dilution factors (1:0, 1:1, 1:2, 1:4 and 1:9). The evaluation of matrix effect using this method was studied for the final extract of SPME for spiked human plasma at a concentration of 2µg/ml of standard PUFAs; results were then compared with that of the absolute matrix effect method.

Supporting Results

2.1.   SPME method development

Wash: Exposure of the coatings to complex biofluidic matrices provides the risk of attachment of particulates and macromolecules into the coating surface. Therefore, optimization of a fast washing step after extraction is crucial for efficient cleaning of the coating surface with minimum loss of analytes. This also helps to minimize the contamination of the final extract and avoid possible ion suppression/enhancement caused by interfering components in electrospray ionization source. Figure S-3 illustrates the effect of different washing approaches on percentage recovery. The evaluation of the wash step in this study indicated that a 10 s immersion of fibers in nanopure water was found to be optimal for efficient cleaning of the coatings after extraction from plasma samples. Extending the washing step or application of any mechanical agitation in this study resulted in a loss of precision and reproducibility due to an inconsistent loss of analytes and observation of higher variation in the results.

Desorption: In order to achieve the most efficient desorption of compounds from the coating and to minimize any remaining trace of compounds, desorption conditions should be optimized. Different compositions and ratios of organic:water phases were compared to find the best desorption solvent (Figure S-4). Results indicated that the 100% acetonitrile solvent resulted in the best recovery and the lowest carryover. According to desorption time profile, the most efficient desorption of the analytes with the lowest carryover was found at a minimum 60 min desorption time at 1500 rpm agitation speed (1 mm amplitude).

Carry over: Efficiency of desorption should also be determined by evaluation of possible carryover. However, considering that it is impossible to evaluate the potential carryover of all macromolecules and metabolites present in a typical biological sample, these biocompatible

SPME devices are recommended for single use for both *in vivo* and *in vitro* applications. This is recommended in order to prevent accidental cross contamination of subsequent samples by any coeluting traces from previous extractions. The amount of carryover of analytes in the coating was evaluated through a second desorption of the same set of fibers used for evaluation of desorption time. Results concluded that a 90 min single step desorption was sufficient to eliminate fatty acids from the fiber, and an analysis of the second desorption did not result in any detectable signals. A desorption efficiency greater than 95% is acceptable for quantitative analysis. Due to the desorption efficiency of the analytes, percentages of carryover were found negligible regardless of type of extraction biological matrix being sampled.

2.2.   **Extraction efficiency of the SPME method in PBS:**

The absolute recovery of FAs from a physiological buffer solution (pH 7.4) using biocompatible fibers was higher than 44% for all cases, which resulted in a significant depletion conditions during the binding study. Coatings with such high fiber constants are preferred for the study of FAs in order to ensure that the amount of ligand extracted by the coating is sufficiently high so that instrumental sensitivity is adequate to determine the extracted amount accurately. The results obtained in the PBS buffer solution as a matrix-free media are shown in table 1. The precision of the proposed system was studied as inter- and intra-day relative standard deviations (RSD) for n = 6 coatings over four experiments. The assay showed good precision (5–12% intra- and 1–6% inter-day RSDs) for the analysis of all nine fatty acids. The matrix match calibration curve was also constructed in PBS in order to determine the linear regression equation and the correlation coefficient (r^2) of the standard calibration line using the least squares method. The linearity of the standard calibration curve was confirmed by plotting the extracted amount (ng) versus spiked amount in plasma. Limit of quantification (LOQ) was determined by analyzing fatty acids in five replicates and verifying the RSD%, which should be smaller than 15%. Data are presented in Table 1.

Table 1 Evaluation of SPME method efficiency for extraction of FAs from physiological buffer solution (pH=7.4)

FA	Absolute Recovery (n=5)	Inter-day RSD (4 trials)	Intra- day RSD (n = 6)	LOD ng/mL	LOQ ng/mL	R^2	Linearity ng/mL
DHA	44±7	8	1	1.5	5	0.9986	10-1500
DPA	76±1	7	1	1	5	0.9958	7-1000
ADR	68±1	5	3	1	5	0.9997	7-1500
DTA	90±6	11	2	1.5	5	0.9973	2-500
EPA	61±10	9	4	3	10	0.9984	10-1500
ARA	55±8	8	6	1.5	5	0.9931	10-1500
ETA	74±3	7	2	1.5	5	0.9999	7-1000
STD	81±8	12	3	5	10	0.9981	5-1000
ALA	63±3	6	2	1	5	0.9970	10.1500

## 2.3. Evaluation of matrix effect using Sample Extract Dilution Method

The sample Extract Dilution method was applied as an additional quantitative method in order to confirm the absence of an absolute matrix effect by proposed SPME method. Therefore, a plot was constructed for each analyte; the x axis reports the dilution factor, and the y axis represents the normalized peak area (peak area of the chromatographic peak multiplied by dilution factor). In the electrospray ionization (ESI) source, the total number of ions per time unit formed is approximately constant; at higher concentrations a competition occurs between all ions to escape from the final droplet surface. Once the compound concentration decreases as a result of sample dilution, this competition decreases concurrently. As a result, the matrix effect on analyte response originated by coeluting compounds can be reduced significantly. [2] When there is no absolute matrix effect, the y axis response remains constant for the entire applied dilution factors, within the experimental error; Figure S-5). The results of this evaluation indicated that in spite of the complexity of the plasma matrix, the final extract obtained from the SPME method required no dilution in all cases. The SPME method resulted in clean final extracts of biological samples due to the isolation of analytes from any interfering matrices. In addition, the application of the biocompatible coatings and washing step aided to prevent transfer of macromolecules (including polysaccharide, proteins, and particulates) and in reducing the possibility of suppression/enhancement of analytes signals during ionization.

Supporting Figure S-1: Examples of XIC chromatograms for SPME-LC-MS analysis of 2µg/mL of fatty acids from human plasma. (TIC 270-335; DHA:327.3, DPA: 329.3, ADR: 332.3, DTA: 333.3, EPA: 301.3, ARA: 303.3, EPA: 305.3, SDA: 275.2 and 10-ALA: 277.2 respectively)
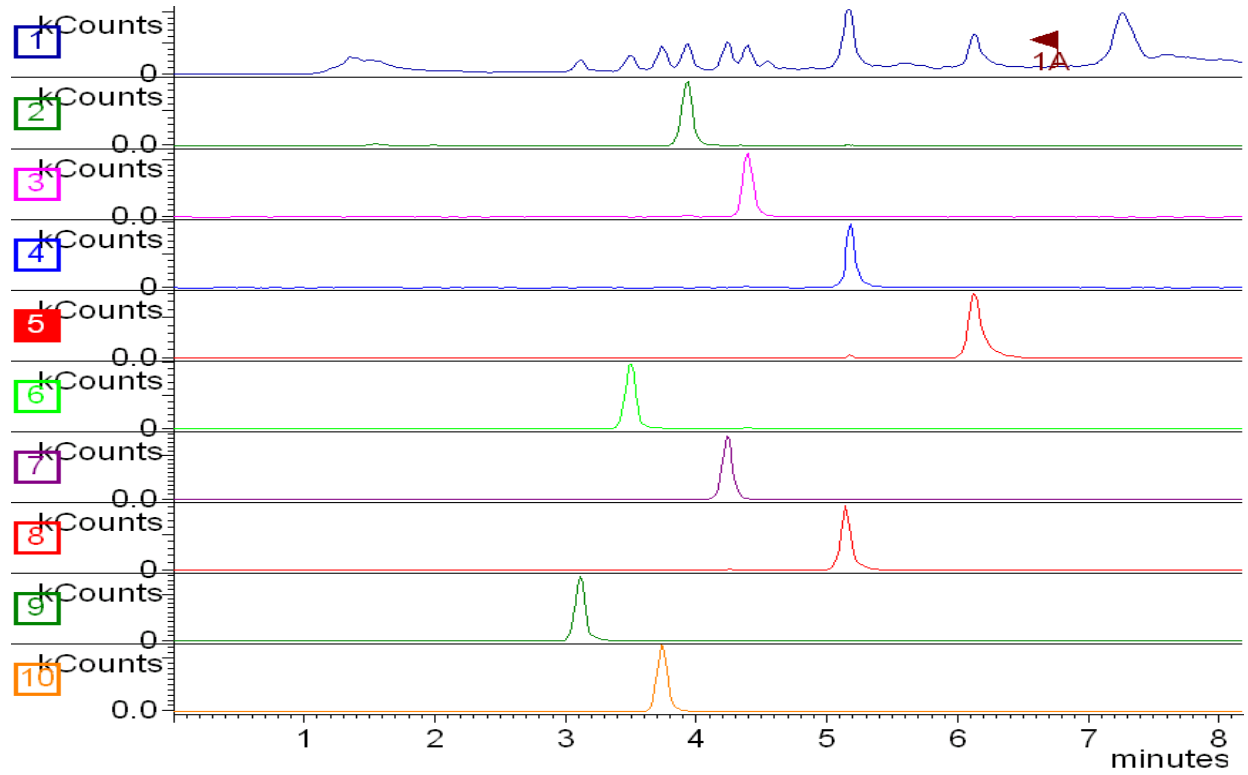
Supporting Figure S-2: Extraction time profile for extraction of (3000 ng/mL) fatty acids spiked in plasma, the second plateau is an unusual observation due to the initiation of protein aggregation around the fiber after 4 hours extraction with aggressive vortex agitation
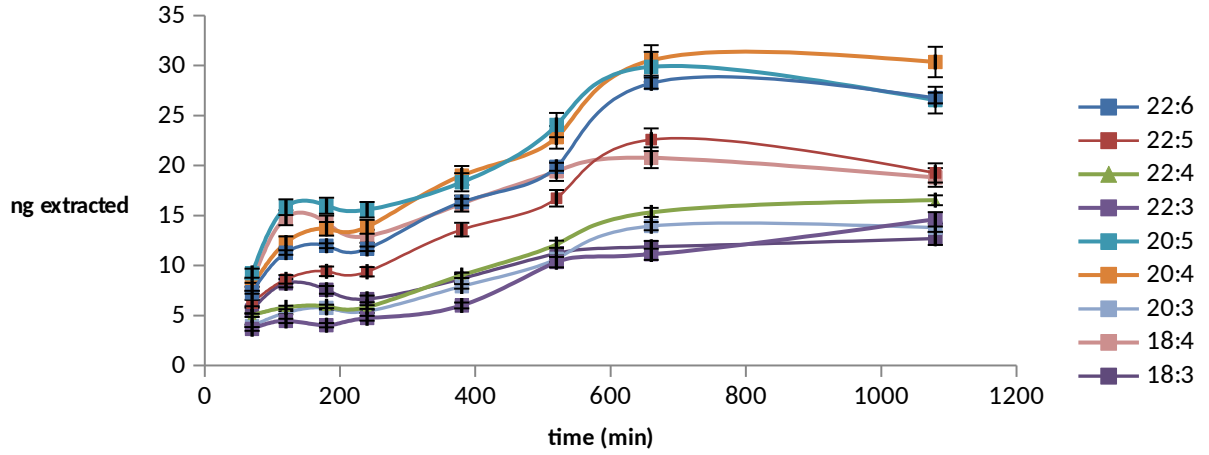
Supporting Figure S-3: Evaluation of analyte loss using different washing strategies, extraction from spiked PBS (30ng/ml)
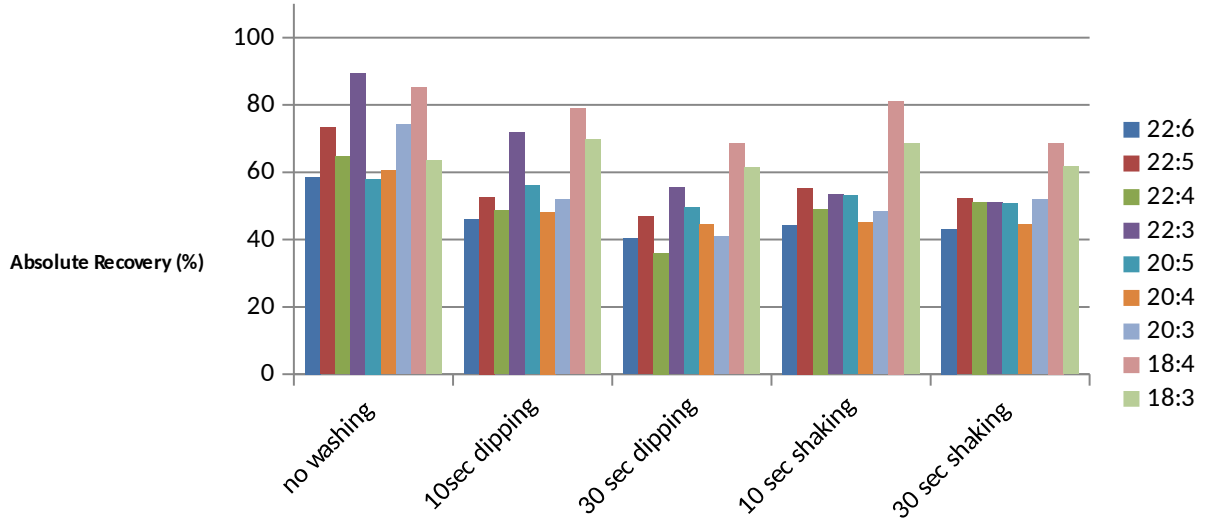
Supporting Figure S-4: Desorption solution composition containing acetonitrile, ACN/MOH/W (40:40:20) plain or with additives of NH$_4$OH (pH 7.8), Formic acid (pH 3.2) or tributhylamin (pH 9.2), extraction from spiked PBS (30ng/ml)
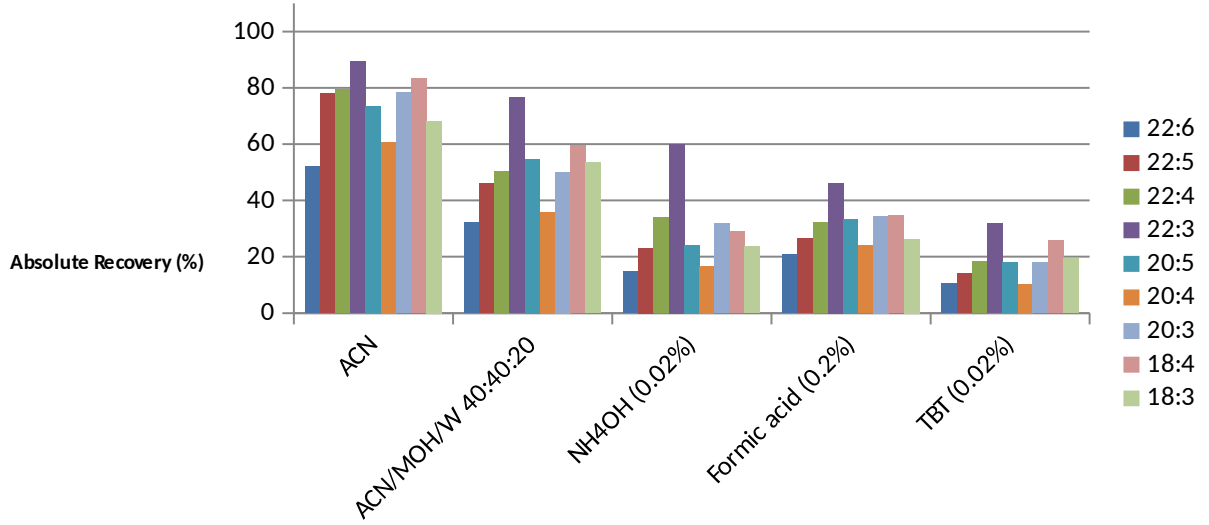
Supportin Figure S-5: Plasma sample dilution effect on normalized ion current intensity for fatty acids extracted from plasma
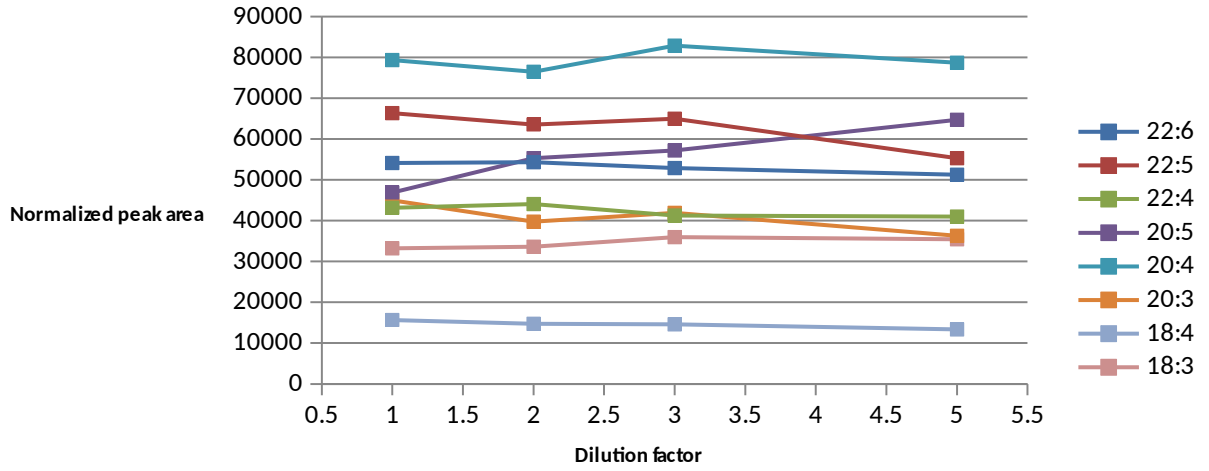
3. References

(1)   Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal. Chem.* **2003**, *75*, 3019–3030.

(2)   Cavaliere, C.; Foglia, P.; Gubbiotti, R.; Sacchetti, P.; Samperi, R.; Lagana, A. **2008**, 3089–3099.