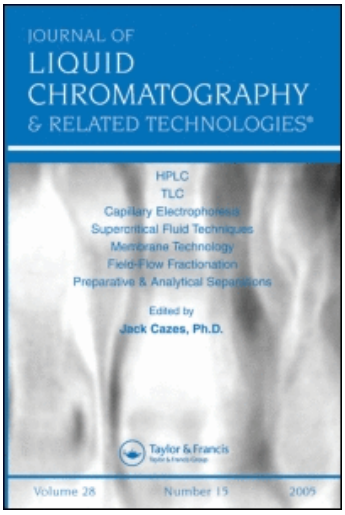

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ISOLATION AND QUANTITATION OF PLASMA LYSOPHOSPHATIDIC ACIDS BY SOLID-PHASE EXTRACTION AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

In this study, a simple and rapid method was developed for isolation and quantitation of lysophosphatidic acid (LPA) molecular species in human plasma. LPA-spiked human plasma (no detectable LPAs) was first isolated and concentrated by Dual-Zone^{TM} C8 solid-phase extraction cartridge. The extracts were then determined by capillary electrophoresis (CE) with indirect UV detection using adenosine monophosphate (AMP) as the UV-absorbing electrolyte.

The separation of LPAs molecular species (myristoyl-, palmitoyl-, stearoyl-, and oleoyl-) was achieved within 13 min. With LPA(D) as internal standard in plasma, the method had linear calibration ranges for LPAs from 0.68 μM to 66 μM, and the correlation coefficients were greater than 0.998.

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This method also had absolute recoveries of LPAs $\geq$ 83% and reproducibilities of area ratios LPA/I.S. (%CV) $\leq$ 7% for plasma samples.

INTRODUCTION

Lysophosphatidic acids (LPAs), comprising several molecular species with various fatty acyl groups (i.e., palmitoyl, stearoyl, oleoyl, linoleoyl, linolenoyl, arachidonoyl, and docosahexarnoyl), have been reported as the potent components of the ovarian cancer activation factor in ascites of ovarian cancer patients (1). LPA is usually not detectable in the plasma of healthy individuals; however, it may be abnormally associated with ovarian cancer and can be detected in plasma from patients with ovarian cancer (2,3). According to Xu et al. (1–3) at the Cleveland Clinic Foundation, the elevated plasma LPAs may be used as biomarkers for early detection of human ovarian cancer.

In general, an analytical method for analyzing molecular species of phospholipid in blood samples is typically a three-step procedure. It includes (a) isolation of total lipid from biological matrix; (b) fractionation of lipid to various phospholipid groups; and (c) separation and analysis of molecular species of individual phospholipids. Liquid-liquid extraction (LLE) was the most commonly used technique to isolate the total lipids from blood sample (4,5). Amberlite resins SPE was reported for the direct extraction of phospholipids from plasma (6). Thin-layer chromatography (TLC), normal-phase high-performance liquid chromatography (HPLC) or solid-phase extraction (SPE) (7) were often used to fractionate the lipids to various groups prior to the detection by gas chromatography (GC) or reverse-phase HPLC (8). Each analytical method might have its own merits; however, the isolation and fractionation were the most time-consuming steps and often suffered from poor recovery.

For LPA analysis, a method using LLE, TLC, and GC was reported (2). More recently, a mass spectrometry-based method and a capillary electrophoresis-based method have been developed (3,9). Although these methods are sensitive and quantitative, the laborious and time-consuming LLE and TLC were still used for sample preparation. Therefore, a simple, rapid, and precise method is needed for sample preparation in LPA analysis.

In this study, an analytical method that uses Dual-Zone^{TM} C8 SPE cartridge for plasma sample preparation and CE technique for separation and detection of whole LPA individual molecular species has been developed. The application of SPE for plasma LPA preparation has significantly reduced the sample preparation time, and improved recovery and precision of the method. This paper is the first report of SPE for plasma LPA preparation.

EXPERIMENTAL

Chemicals and Solutions

Lysophosphatidic acids (synthetic, purity > 99%) [1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphate monosodium salt, LPA(M); 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate monosodium salt, LPA(P); 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate monosodium salt, LPA(S); 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate monosodium salt, LPA(O); and 1-decanoyl-2-hydroxy-*sn*-glycero-3-phosphate monosodium salt, LPA(D), internal standard for the CE analysis]; and 1,2-dipalmitoyl-*sn*-glycero-3-phosphate monosodium salt, PA(P) (synthetic, purity > 99%, internal standard for the SPE recovery studies) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). (−)-Adenosine 5′-monophosphate monohydrate (AMP) (99%), phosphoric acid (99.999%), and sodium hydroxide (99.99%) were obtained from Aldrich (Milwaukee, WI). Human plasma (Cat. No. P9523), HPLC grade methanol, acetonitrile were obtained from Sigma (St. Louis, MO). Boric acid (electrophoresis purity reagent) was obtained from Bio-Rad Laboratories (Richmond, CA). HPLC grade glacial acetic acid was obtained from J. T. Baker (Phillipsburg, NJ). Sodium phosphate was obtained from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Chloroform, ammonium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was obtained from a Barnstead/Ther-Thermolyne NANOpure system (Dubuque, IA).

Buffers and Standards

Stock buffer solutions were 50 mM AMP and 100 mM boric acid prepared in deionized water and adjusted to the desired pHs with 1 M NaOH. The stock buffer solutions were filtered through 0.45-μm cellulose acetate membrane syringe filters (Alltech Associates, Inc., Deerfield, IL) before use. CE separation buffer solutions (5 mM AMP and 10 mM boric acid) were prepared by 10-fold dilution of the stock buffer solutions with methanol, and degassed in an ultrasonic bath for 30 min (100 mL each) before use.

5 mM stock standard solutions of LPA(S), LPA(O), LPA(P), LPA(M), PA(P), and LPA(D) were prepared in $CH_3OH/CHCl_3$ (2 : 1, v/v) individually. From these stock solutions, 1 mM mixture standard of LPA(S), LPA(O), LPA(P), LPA(M), 1 mM PA(P), and 1 mM LPA(D) were prepared in $CH_3OH/CHCl_3$ (2 : 1, v/v) prior to use. As standard solutions were prepared by appropriate dilution of 1 mM mixture standard with 5% separation buffer in CH_3OH/H_2O (9 : 1, v/v), plasma standards were prepared by dilution of 1 mM mixture standard with blank human plasma.

For the recovery study of SPE procedure, PA(P) was used as the internal standard, which was added to the extract prior to the CE analysis. For the CE analysis, LPA(D) was chosen as the internal standard, which was added to the samples prior to the SPE procedure. For plasma LPA analysis, 0.5 mL plasma sample together with 15 μL of 1 mM I.S. LPA(D) was mixed with 1 mL of 0.1 M phosphate buffer at pH 3 and 100 μL CH_3CN prior to SPE.

Solid-Phase Extraction

SPE was performed using a 12-port Visiprep SPE Vacuum Manifold (Supelco, Bellefonte, PA). Three kinds of cartridges: Supelco Hisep SPE cartridges (500 mg, 3 mL), Diazem Dual-Zone SPE C_8, and C_{18} cartridges (200 mg, 3 mL, MetaChem Technologies, Torrance, CA) were examined for plasma LPA preparation. Before applying samples, each cartridge was conditioned with 4 mL CH_3OH, followed by 2 mL of 0.1 M phosphate buffer at pH 3 to equilibrate the cartridge. The plasma sample was then loaded to the cartridge at a slow flow rate (< 0.1 mL/min) to ensure the complete interaction between LPAs and packing material. The cartridge was washed with 2 mL of 0.1 M phosphate buffer (pH 3), followed by 1 mL of H_2O, and then dried for 10 min under N_2 gas using 12-port Visidry Drying Attachment (Supelco, Bellefonte, PA).

The analytes of interest were eluted from the cartridge with 2 mL of $CH_3OH/CH_3CN/NH_4OH$ (70 : 30 : 5, v/v/v) solvent unless otherwise specified, and dried under N_2 gas. Prior to CE analysis, the dry powder was reconstituted with 0.5 mL of 5% separation buffer in CH_3OH/H_2O (9 : 1, v/v) or less volumes if pre-concentration was desired.

CE Instrumentation

Open tubular fused-silica capillary (75 μm i.d., 354 μm o.d.) was obtained from Polymicro Technologies (Phoenix, AZ). The uncoated capillary was 47 cm in length (40 cm to the detection window). This capillary was mounted in a P/ACE cartridge that was connected to a temperature control system. The new capillary was conditioned, sequentially, with 1.0 M NaOH for 1 hr., deionized water for 20 min, CH_3OH for 20 min, and separation buffer for 30 min. As a daily routine, the capillary was rinsed with 0.1 M NaOH for 10 min, deionized water for 5 min, and the separation buffer for 15 min.

A Beckman (Fullerton, CA) P/ACE 2200 system and an IBM PC with System Gold software (Version 8.1) were used for CE separation and data acquisition. On-line indirect UV detection was performed at 260 nm, and the separation temperature was maintained at 25°C. Samples were introduced into the

capillary by pressure injection at 3.45 kPa for 4 s. Separations were carried out under applied potential of 25 kV at the normal polarity (the cathode was placed at the outlet of the capillary). Between runs, the capillary was rinsed with 0.1 M NaOH for 1 min and the separation buffer for 2 min.

RESULTS AND DISCUSSION

Plasma Sample Preparation

The LLE and TLC procedures for sample preparation developed by Xu et al. (2) have been used in our previous work for the extraction and fractionation of serum LPAs (9). It was found that the recoveries of LPAs were remarkably low with large variations. The TLC procedure for the separation of LPA sub-group from other lysophospholipids, especially LPI, suffered from poor resolution and might result in sample contamination (9,10). To reduce the sample preparation time and improve the recovery with high reproducibility, as well as to eliminate the contamination from other lysophospholipids, a SPE procedure for plasma LPA preparation was developed in this study.

SPE Cartridges

Supelco Hisep (500 mg), Diazem Dual-Zone C8 (200 mg), and C18 (200 mg) SPE cartridges were compared for the recoveries of plasma LPAs. The results are shown in Table 1. Using methanol as an elution solvent, the highest absolute recoveries (81–96%) of plasma LPAs standards were achieved for all the species using Diazem Dual-Zone^{TM} C8 cartridges. According to the manufacturer, Dual-Zone^{TM} SPE cartridges employ packing materials that have hydrophilic outer surface and hydrophobic inner surface. Plasma samples can be loaded directly onto the cartridge where macromolecules are excluded by the pore size and the electrostatic repulsion of the outer surface of packing material, and small hydrophobic molecules are absorbed by the inner hydrophobic surface. Table 1 showed that Dual-Zone^{TM} C18 cartridge, which is more hydrophobic gave ca. 5% lower recoveries of LPAs than those of Dual-Zone^{TM} C8 cartridge. This is due to LPAs being amphiphilic molecules that have long fatty acyl tails (C14–18) and negatively charged phosphate heads. When these molecules interacted with Dual-Zone^{TM} SPE cartridge, they showed stronger adsorption on the C8 cartridge than on the C18. In the case of Supelco Hisep cartridge, it only gave about 50% recoveries of plasma LPAs. The poor recoveries of the Supelco Hisep cartridge were not due to the insufficient elution of LPAs, since a larger volume of methanol (3 or 4 mL) for elution showed no improvement on the

Table 1. The Absolute Recoveries of Plasma LPAs from Three Kinds of SPE Cartridges[a]

	Diazem Dual-Zone C_{18}		Diazem Dual-Zone C_8		Supelco Hisep	
Compound	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
LPA(S)	78	6	81	4	59	1
LPA(O)	91	1	96	1	54	5
LPA(P)	86	6	91	4	55	6
LPA(M)	87	2	95	2	51	4
LPA(D)	86	4	90	1	14	5

[a]The concentration of LPAs was 50 μM each. Each datum point was based on two measurements.

recoveries. According to Kimata et al. (11), the order of hydrophobicity for these cartridges is as follows: Dual-Zone^{TM} C18 > Dual-Zone^{TM} C8 ≫ Supelco Hisep. It seems that Supelco Hisep cartridge does not have enough hydrophobicity to retain the analytes. Since Dual-Zone^{TM} C8 (200 mg) showed the best recoveries for plasma LPAs, it was chosen for the subsequent plasma LPAs extraction.

Effect of Buffer pH Value on Electrophoretic Separation

In our previous study (9), the optimum conditions for CE separation of LPAs were 5 mM AMP and 10 mM boric acid at pH 5.4 in CH_3OH/H_2O (9 : 1 v/v). Under these conditions, the standard LPAs could be separated with very high efficiency; however, due to the matrix effect the plasma LPAs experienced poor resolution after SPE (Figure 1a).

To overcome this problem, various buffer pHs for the CE separation were investigated. Figures 1b and 1c showed the electropherograms of the blank plasma and plasma spiked LPAs under buffer pH 6.0 and 7.0. The matrix interferences could be completely resolved from LPAs at pH 6.0 and 7.0. However, buffer pH not only affected the resolution and the migration times of LPAs, but also influenced the mass to charge ratio of the interfering compounds. Based on the observed shift of the interfering peaks, these matrix interfering compounds must have multiple ionizable groups that could be affected by the chosen pHs.

As seen in Figure 1c, most of LPA peaks split into one major and one minor peak under pH 7.0. This was probably caused by the dissociation of the second hydroxide group of phosphate moiety of LPAs, which reportedly have pK_as of

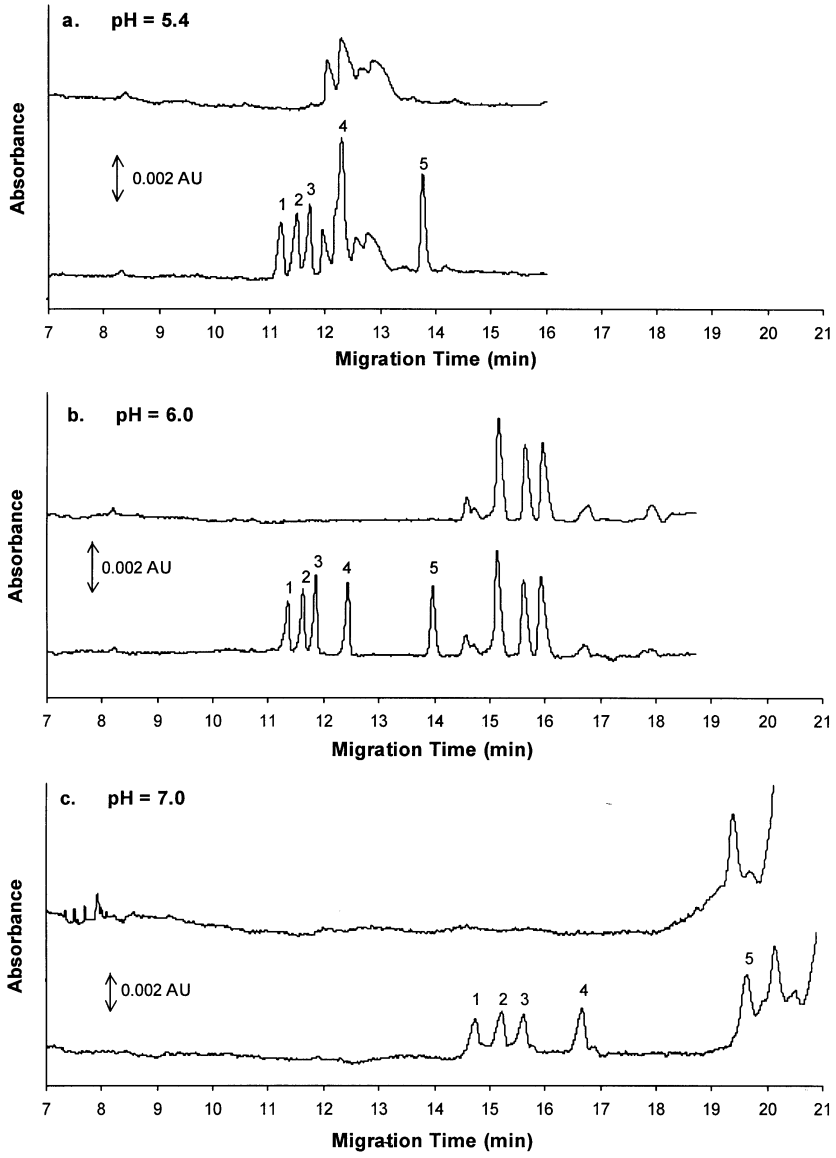

Figure 1. The effect of buffer pH on the separation of plasma LPAs after SPE. Buffer composition: 5 mM AMP and 10 mM boric acid in CH_3OH/H_2O (9:1 v/v). Peak identities: 1. LPA(S); 2. LPA(O); 3. LPA(P); 4. LPA(M); 5. I.S. LPA(D). The upper traces of a, b, and c were from the control blank plasma and the bottom traces were from the control blank plasma spiked with the LPA standards.

$8 \sim 9$. Therefore, the optimum pH for the CE separation of plasma LPAs after SPE would be pH 6.0.

Elution Solvent

Methanol is a common choice of solvent to fractionate phospholipids from total lipid by SPE due to its solvent strength. However, if methanol was used alone as the elution solvent, the recoveries were not consistent with various LPA species. For instance, the recovery of LPA(S) was less than 80%, whereas the other LPAs were greater than 80%.

In this work, the mixtures of methanol and acetonitrile with weak acid or weak base [i.e., $CH_3OH-CH_3CN-Hac$ (70 : 30 : 5) and $CH_3OH-CH_3CN-NH_4OH$ (70 : 30 : 5)] were investigated as the elution solvents to improve the recoveries of plasma LPAs and consistency of the SPE procedure. The weak acid or base added to the elution solvent was an attempt to increase the interaction between solvent and LPAs. The experiments revealed that weak base additive could improve the recovery of LPA(S) to $> 80\%$, which was probably due to the reduced interaction between sorbent and LPAs at higher pH values. The elution solvent, $CH_3OH-CH_3CN-HAc$ (70 : 30 : 5), not only did not improve the recoveries of LPAs, but, further reduced them to $60\% \sim 70\%$. Moreover, the reduction of the elution solvent volume from 3 mL to 2 mL did not significantly affect the LPA recoveries. Therefore, 2 mL of $CH_3OH-CH_3CN-NH_4OH$ (70 : 30 : 5) was chosen as the elution solvent for method validation.

Plasma Sample Additives

To improve the recoveries of plasma LPAs on SPE cartridges, plasma sample additives were used. In this work, 1 mL of 0.1 M phosphate buffer (pH = 3) was used for the dilution of 0.5 mL plasma sample, which could protonate the phosphate groups of LPAs and promote the hydrophobic interaction between the sorbent and LPAs. Furthermore, 100-μL acetonitrile (or methanol) was added to the plasma samples to denature the plasma protein and release the protein bound LPAs. The experiments indicated that both additives could improve the recoveries of LPAs (data not shown). In the case of organic additives, acetonitrile worked better than methanol, because the latter could more easily cause precipitation of plasma proteins.

Internal Standards

In our previous work, LPA(D) had been used as the internal standard for the method calibration (9). In this work, PA(P) was used as the internal standard to

evaluate the recoveries of plasma LPAs, including LPA(D) by SPE. PA(P) was added to the extract of SPE prior to CE analysis. This internal standard could correct the variation by CE detection. Since PA(P) did not show up in the extract of SPE, it was not our concern whether it was present in the original samples or not. From Table 2, it was known that the recovery of LPA(D) was compatible with the recoveries of other LPAs by SPE. Therefore, LPA(D) was chosen as the internal standard for the method calibration in this work as well.

Recoveries and Method Comparison

The absolute recoveries of plasma LPAs from Dual-Zone^{TM} C8 SPE cartridges were summarized in Table 2. Using the developed SPE procedure for sample preparation, recoveries of 83–93% could be obtained for LPA(S), LPA(O), LPA(P), LPA(M), and LPA(D) from human plasma samples. In comparison to the conventional LLE/TLC method for plasma LPAs preparation (9), the SPE procedure is superior in terms of recovery, reproducibility, sample handling, and processing time (Table 3).

Analytical Performance

In this work, the complete separation of LPAs and I.S. LPA(D) by CE could be done within 15 min under buffer pH 6 (Figure 1b), and the analysis of plasma LPAs could be accomplished in less than 2.5 hours. The intra- and inter-assay repeatabilities were evaluated and the results were summarized in Table 4. The repeatabilities of the area ratio (the peak area of LPA vs. the peak area of I.S.) calculated as %CV were $\leq 7\%$. The internal calibration was used for the

Table 2. The Absolute Recoveries of Plasma LPAs by SPE Using Diazem Dual-Zone^{TM} C8 Cartridge

	20 μM LPA[a]		50 μM LPA[b]	
Compound	Recovery (%)	CV (%)	Recovery (%)	CV (%)
LPA(S)	89	1	85	4
LPA(O)	80	3	83	2
LPA(P)	90	2	93	3
LPA(M)	90	3	85	4
LPA(D)	88	3	85	4

[a]20 μM PA(P) was added before CE analysis (n = 3).
[b]50 μM PA(P) was added before CE analysis (n = 3).

Table 3. The Comparison of LLE-TLC Method with the Developed SPE Method for Preparation of Plasma LPAs

	Recovery[a]				Plasma Sample Preparation Time
	LPA(S)	LPA(O)	LPA(P)	LPA(M)	
Conventional method[b]	23% (13% CV)	32% (9% CV)	37% (1% CV)	36% (8% CV)	6.5 hr
Our method[c]	85% (4% CV)	83% (2% CV)	93% (3% CV)	85% (4% CV)	2 hr

[a]Results were obtained by CE detection.
[b]The concentration of LPAs and I.S. LPA(D) was 50 μM each. The I.S. was added before CE analysis (n = 2).
[c]The concentration of LPAs and I.S. PA(P) was 50 μM each. The I.S. was added before CE analysis (n = 3).

Table 4. Intra- and Inter-Assay Repeatability by Area Ratio[a]

	Intra-Assay		Inter-Assay	
Compound	LPAs/I.S.	CV (%)	LPAs/I.S.	CV (%)
LPA(S)	0.803 ± 0.020	3	0.797 ± 0.035	4
LPA(O)	0.870 ± 0.013	2	0.875 ± 0.015	2
LPA(P)	0.785 ± 0.013	2	0.768 ± 0.013	2
LPA(M)	0.946 ± 0.066	7	0.960 ± 0.024	3

[a]The concentrations of LPAs and I.S. LPA(D) in plasma were 50 μM; n = 4.

Table 5. The Internal Calibration Equations and Limits of Detection (LOD) of Plasma LPAs

	Calibration Curve[a]		LOD[b]
Compound	Least Square Equation	R^2	μM
LPA(S)	$y = 0.0259x - 0.0146$	0.9995	0.56
LPA(O)	$y = 0.0297x - 0.0165$	0.9982	0.51
LPA(P)	$y = 0.0259x - 0.0078$	0.9993	0.53
LPA(M)	$y = 0.0313x - 0.0221$	0.9996	0.50

[a]The concentrations of LPAs were from 0.68 to 66 μM, and I.S., LPA(D), was 31 μM (n = 3).
[b]Calculated by a signal-to-noise ratio of 3 from the calibration curves with 10-fold sample pre-concentration.

quantitation of LPAs. The regression parameters and the limits of detection were given in Table 5. The calibration curves had linear ranges from 0.68 to 66 μM with three injections for each of the six concentrations (0.68, 5.4, 11, 23, 46, and 66 μM). The lowest concentration of the calibration curves had an 8.3-fold pre-concentration. The correlation coefficients were greater than 0.998. The limits of detection calculated as 3 times of signal-to-noise ratio were ca 0.5 μM by 10-fold pre-concentration, which was achieved by reconstituting the dry powder of the extract with 50-μL solution.

CONCLUSION

A simple and quantitative method has been developed for the determination of LPAs in human plasma. This method uses SPE for plasma LPAs isolation and

CE for LPAs separation and detection. It has excellent analytical performances, low limits of detection, and high recoveries of LPAs. This report is the first paper on SPE for plasma LPAs preparation. It is a useful method for analyzing molecular species of LPAs in human plasma.

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