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Separation of *Cis-Trans* Phospholipid Isomers using Reversed Phase LC with High Resolution MS Detection

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Abstract

The increased presence of synthetic *trans* fatty acids into western diets has been shown to have deleterious effects on physiology and raising an individual's risk of developing metabolic disease, cardiovascular disease, and stroke. The importance of these fatty acids for health and the diversity of their (patho) physiological effects suggest that not only should the free *trans* fatty acids be studied, but that monitoring the presence of these fats into the side-chains of biological lipids, such as glycerophospholipids, is also essential. We developed a high resolution LC-MS method that quantitatively monitors the major lipid classes found in biospecimens in an efficient, sensitive and robust manner while also characterizing individual lipid side-chains through the use of HCD fragmentation and chromatographic alignment. We herein show how this previously described reversed phase method can baseline separate the *cis-trans* isomers of phosphatidylglycerol and phosphatidylcholine (PC) with two 18:1 side chains, in both positive and negative mode, as neat solutions and when spiked into a biological matrix. Endogenous PC (18:1/18:1) *cis* and PC (18:1/18:1) *trans* isomers were examined in mitochondrial and serum profiling studies, where rats were fed diets enriched in either *trans* 18:1 fatty acids, or *cis* 18:1 fatty acids. In this study, we determined the *cis:trans* isomer ratios of PC (18:1/18:1) and related this ratio to dietary composition. This generalized LC-MS method enables the monitoring of *trans* fats in biological lipids in the context of a non-targeted method, allowing for relative quantitation and enhanced identification of unknown lipids in complex matrices.

Keywords

Cis-Trans stereoisomers; liquid chromatography; mass spectrometry; lipidomics; dietary macronutrients

INTRODUCTION

Trans fatty acids are unsaturated fatty acids that have at least one carbon double bond in the *trans* configuration. *Trans* fatty acids are not endogenously synthesized by humans, and their main source is food intake. Industrially-produced *trans* fats are formed during the partial hydrogenation of vegetable oil that changes *cis* configuration of double bond(s) to *trans*. These species contribute 4–12% of dietary fat intake in modern U.S. diets.^{1, 2} The increased presence of synthetic *trans* fatty acids into western diets has been shown to have deleterious effects on physiology and to raise an individual's risk of developing metabolic disease, cardiovascular disease, and stroke.^{3–5} As one example, dietary *trans* fat intake conveys approximately double the atherosclerotic risk when directly compared to saturated fats.^{6, 7} This increase in risk is caused, in part, because *trans* fats simultaneously raise low density

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lipoprotein (LDL) and lower high density lipoprotein (HDL), which dramatically increases the risk of coronary heart disease, whereas saturated fatty acids (SFA) are only known to lower LDL.⁶⁻⁸ In addition, *trans* fatty acids reduce the availability of healthy polyunsaturated omega-3 fatty acids such as eicosapentaenoic acid [FA(20:5)] and docosahexaenoic acid [FA(22:6)] for human metabolism by impeding both delta-6 and delta-3 desaturation and elongation enzymes.⁹ Analysis of Nurse's Health Study data revealed a positive correlation between *trans* fat intake and risk for diabetes development¹⁰. Due to these and other related effects, monitoring the presence of these species into biological lipids should be of interest in the clinic when assessing patient overall health and disease risk, in epidemiology studies when interpreting population-level risk factors, as well as in the laboratory, e.g., when performing mechanistic studies in animals and in cell cultures.

Analysis of *cis-trans* isomers is usually done via lipid hydrolysis to free fatty acids (FA), esterification of those FAs, and subsequent gas chromatography (GC) based separation. This technique is limiting in that it only measures the free fatty acids, and thus loses the linkage with the lipid categories from which they have originated. Understanding the origin of the *trans* fat, e.g. glycerolipid, glycerolphospholipid or sphingolipid, is expected to give greater insight into the mechanism of disease risk than just the presence of the free FA alone.

Reversed phase (RP) LC separation with MS detection has been used widely in lipid analysis and has proven to be a sensitive and robust technique.¹¹⁻¹⁵ This method provides efficient intra- and inter-class separations. Until now, however, its application to *cis-trans* isomer analysis has been limited.¹⁶ Previous studies have utilized MS-directed LC fractionation of free FA chromatographic regions which then require further analysis of the resulting fractions by GC to fully characterize the multiple species eluting in those areas and the stereochemistry of each species.¹⁷ An alternative approach has focused on silver ion HPLC methods^{18, 19}, which yield greater stereospecific separations, but these methods are not readily amendable to MS detection, and they have only been applied to hydrolyzed FAs, not intact lipids.

Reversed phase LC separates lipids based on lipid head group polarity, an inter-class property, and the carbon side chain hydrophobicity, an intra-class attribute. In lipid chemistry, the incorporation of double bonds into the hydrocarbon side-chain affects the properties of the lipid by lowering the melting point, altering solubility, and creating a much less flexible molecule. These less flexible species are less retained on RP chromatographic particles and therefore are separated from their saturated counterparts. We hypothesized that the same mechanism of separation should be applicable to *cis-trans* isomers, where the *trans* isomer behaves, chromatographically, more like a straight saturated molecule in comparison to its bent *cis* equivalent. The structural differences of these two stereoisomers can be seen in Figure 1. Ideally an LC-MS method that can separate multiple classes of lipid compounds, efficiently and robustly, would also yield stereospecific information about the individual lipids as well.

Non-targeted RP LC-MS lipidomics profiling is used to simultaneously detect species across multiple lipid classes without any deliberate bias. This type of analysis has the ability to yield comprehensive, quantitative, and reproducible analytical data in an efficient and robust manner from any given biological sample,^{11-15, 20, 21} and was used to profile rats fed diets that varied by macronutrient content, both the fat and glycemic index, with two of the fat sources being either a *trans* or *cis* 18:1.^{11, 12, 15} Herein, we show how endogenous serum and mitochondrial PC (18:1/18:1) *cis-trans* isomers were able to be quantitatively profiled across all animals (n=192) in the investigation and *cis/trans* isomer ratios of PC (18:1/18:1) compared to directly relate fatty acid presence to dietary composition. This analysis provides

a way to monitor *trans* fats in intact biological lipids, as a means to study their effect on total lipid regulation and overall mitochondrial physiology.

MATERIALS AND METHODS

Chemicals

LC-MS grade acetonitrile (ACN), methanol (MeOH), and isopropanol (IPA), as well as HPLC grade dichloromethane (DCM) and dimethyl sulfoxide (DMSO), were purchased from Fisher Scientific (Pittsburg, PA). Ammonium formate, *tert*-butyl alcohol (*t*-BuOH) azobis-isobutyronitrile (AIBN), methyl oleate (MO), and benzenethiol (PhSH) were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) PG (18:1/18:1)-Cis, 1,2-diacyldioyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) PG (18:1/18:1)-*Trans*, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) PG (18:0/18:2), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine PC (18:1/18:1) cis and 1,2-diacyldioyl-*sn*-glycero-3-phosphocholine PC (18:1/18:1) *Trans* were purchased from Avanti Polar Lipids (Alabaster, AL). For both sets of standards, both double bonds in the cis-*trans* pairs are either cis or *trans*, there are no mixed-acyl standards.

Preparation of Lipid Standards

Stock solutions were prepared by dissolving lipid standards in DCM:MeOH (2:1 v/v) at concentrations ranging from 10–50 mg/mL and were stored at –20°C. Working solutions were diluted in ACN/IPA/H₂O (65:30:5 v/v/v) to 1 µg/ml prior to spiking studies or LC-MS analysis.

Dietary Study

Male Fisher 344 x Brown Norway F₁ (FBNF1) rats (n=8), aged 7–9 weeks, were fed *ad libitum* one of 24 isocaloric diets that differed in fat and carbohydrate composition. This approach yielded a total of 192 animals in the study. The diets were comprised of six different fat groups, with the major constituent of each being either saturated fats (SFAs), *trans* fats (*Trans*), monounsaturated fats (MUFAs), or one of 3 groups of polyunsaturated fats (PUFAs), which vary in the ω-6/ω-3 PUFA ratios. Each type of fat was combined with one of 4 carbohydrate groups that varied based on sucrose content. The fat, carbohydrate and protein percentages in all diets were held consistent at 5, 66, and 20 (w/w), equivalent to 12, 68, and 21 (kcal %). Body weights and food composition were measured twice a week and rats were sacrificed after 8 weeks and their liver mitochondria were isolated^{11, 15, 22} and serum was collected. Details of their husbandry and diets will be presented elsewhere (manuscripts in preparation).

Lipid Extraction

Immediately before extraction, each aliquot of mitochondria (containing 1 mg of protein) was dissolved in 40 µL DMSO and the membranes were disrupted by sonication. Mitochondria and serum pool samples were created by combining aliquots from each rat (n=192) and these samples were used for quality control (QC), lipid identification, and standard spiking studies.

Lipids were extracted according to the method of Bligh and Dyer²³, substituting DCM for chloroform²⁴ as described previously^{11, 15}. First, 30 µL of internal standard was added to each 30 µL sample (either mitochondria or serum), followed by 190 µL of MeOH. Next, 380 µL of DCM was added, the sample was again vortexed for 20 seconds and 120 µL of water was added to induce phase separation. The samples were then vortexed for 10 seconds and allowed to equilibrate at room temperature for 10 minutes before centrifugation at 8000 *g* for 10 minutes at 10 °C. A total of 370 µL of the lower lipid-rich DCM layer was then

collected and the solvent evaporated to dryness under vacuum. Samples were reconstituted in 300 μ L of ACN/IPA/H₂O (65:30:5 v/v/v) containing PG (17:0/17:0) at a concentration of 5 μ g/mL before LC-MS analysis.

Free Radical Induced Cis-Trans Biotransformation

Thiyl radical induced isomerization was performed using a slightly modified method of Chatgililoglu et. al²⁵, where a 1.5 mM solution of MO, PC (18:1/18:1) cis and PG (18:1/18:1) cis in *t*-BuOH were incubated separately with 0.75 mM PhSH and 0.3 mM AIBN at 71°C for 100 minutes. In addition to 3 replicates of each standard, three blanks which included only a *t*-BuOH solution without any standards were also prepared. After incubation, 1 μ L of each of these 12 samples were diluted in 1 mL of ACN/IPA/H₂O (65:30:5 v/v/v) before LC-MS analysis.

LC-MS Analysis

Details of the LC-MS method and SIEVE data analysis as applied to lipid profiling have been described previously^{11, 12, 15}. In the current study, PG (18:1/18:1) Cis-*Trans* isomer standards were analyzed on 3 different columns, an Ascentis Express C₁₈ 2.1 $\times$ 150 mm 2.7 μ m column (Sigma-Aldrich, St. Louis, MO), a Waters CSH C₁₈ 2.1 $\times$ 150 mm 1.7 μ m column (Waters, Milford, MA) and a Phalanx C₁₈ 2.1 $\times$ 150 mm 3 μ m column (Nest Group, Southborough, MA), all connected to the same Thermo Fisher Scientific PAL autosampler, Accela quaternary HPLC pump and an Exactive benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray ionization (HESI) probe. Mobile phase A in the chromatographic method consisted of 60:40 Water:ACN in 10mM ammonium formate and 0.1% formic acid and mobile phase B consisted of 90:10 IPA:ACN also with 10mM ammonium formate and 0.1% formic acid. The spray voltage was set to 3.5 kV, whereas the heated capillary and the HESI probe were held at 250 °C and 350 °C, respectively. The sheath gas flow was set to 25 units and the auxiliary gas set to 15 units. The MS was tuned by direct infusion of PG (17:0/17:0) in both positive and negative mode and external mass calibration was performed using the standard calibration mixture from ThermoFisher according to their protocol approximately every five days. These conditions were held constant for both positive and negative ionization mode acquisitions, on all columns tested, and for all experiments performed including mitochondrial and standard biotransformation studies.

The PG standards, PG(18:1/18:1)-cis, PG(18:1/18:1)-*trans* and PG(18:0/18:2) were all run as neat solutions, alone, as a mixture, or spiked into a rat serum pool lipid extract. 10 μ L was injected, and the chromatography was run at 260 μ L/min and 55° C, unless otherwise noted. All LC-MS profiling experiments, including the dietary study and the mitochondrial and STD biotransformation samples, were performed on the Ascentis Express column and analyzed using the MS label free differential analysis software package SIEVE v 1.3 (Thermo Fisher Scientific and Vast Scientific, Cambridge, MA). For the endogenous PC (18:1/18:1) cis-*trans* isomer quantitation studies presented below, SIEVE parameters were altered from our conventional profiling parameters^{11, 15}, to focus on those that would only quantitatively monitor the *m/z* of interest over the specific portion of the chromatogram during which these isomers elute. Specifically, frames were only built for ions in the *m/z* range of 786–787, which represents the [M+H]⁺ ion for PC (36:2), and the RT range of 14.0–16.5 minutes. It is important to note that, under these parameters, SIEVE quantitates over a full chromatographic minute by centering on the apex of the peak and including 30 seconds on either side. These restrictions allowed for the much less intense *trans* peak to be robustly assessed across all samples in our dietary study.

RESULTS AND DISCUSSION

Separation of Cis-Trans PG (18:1/18:1)

To test the hypothesis that *cis-trans* phospholipid isomers could be studied by our RP LC-MS profiling method, we used *cis-trans* PG (18:1/18:1) standards as a proof of concept. Figure 1 shows the structural differences in these standards due to the change in stereochemistry around each double bond. The more linear structure of the *trans* isomer should promote enhanced retention in RP separation. Previous mitochondria^{11, 15} and serum¹² LC-MS lipidomic profiling studies from our laboratory were done using an Ascentis express C₁₈ column where the reduced diffusion path of the fused-core C₁₈ particle is known to provide sharper peaks and enhanced separations at higher flow rates in comparison to fully porous particles.¹³ To determine if any possible *cis-trans* separation observed was an effect of the reduced diffusion path of the Ascentis Express column used previously, we also assessed a Waters 1.7 μ m CSH UPLC column known to give enhanced separation efficiency and a standard fully porous 3 μ m C₁₈ particle column from Phalanx. The Waters CSH technology adds a low level charge to the particle in order to enhance the separation of basic molecules in low ionic strength buffers, in addition to increasing loading capacity. Additionally, although being fully porous, the smaller particle size of 1.7 μ m will also have reduced diffusion paths in comparison to 3 μ m C₁₈ particles. All three columns had an inner diameter of 2.1 mm and were 150 mm in length. It was our working hypothesis that any of the column particle differences discussed could provide either altered or enhanced separation of the *cis-trans* isomers.

Figure 2 shows the separation of a 1 μ g/mL mixture of the *cis-trans* isomers as a neat solution and detected via high resolution full scan MS. Panels A–C represent extracted ion chromatograms (XICs) with a 3ppm mass window of m/z 773.5333 representing the [M-H]⁻ ion of the molecule for the Water's column, the Ascentis express column, and the Phalanx column respectively. From the chromatograms in Figure 2, it is clear that the separation mechanism is not affected by particle size, diffusion path or charged state enhancement provided by the different columns. The only differences seen were in relation to compound retention time and peak width at half height ($W_{1/2}$), and these differences can be explained by the changes in particle size affecting the pressure in the system.

In past experiments,^{11, 12, 15} the Ascentis express column was run at 55°C and 260 μ L/min, which yields pressures between 150 and 200 bar at the low and high portion of our gradient respectively. In order to get the Phalanx column to pressures nearing those, without severely affecting the ionization efficiency of the MS, we eliminated the heat and ran the column at 380 μ L/min. Even at these conditions, the elution was still significantly slower on the Phalanx column in comparison to the Waters or Ascentis columns (Figure 2). Additionally, because the Phalanx column provided a greater particle surface area with which the PG molecules can interact, both the *cis* and *trans* $W_{1/2}$ were wider at 8.4 seconds compared to the 6 second wide peaks observed for the other two columns tested.

The very small particle size of the Waters column increased the pressure in the system dramatically. Since we are not utilizing an ultra high pressure LC (UPLC) system, we had to keep overall pressure at less than 400 bar, and because of this, the enhanced flow rates and shorter analysis time available for the Waters column could not be utilized. Figure 2 shows, that even under sub-optimum chromatographic conditions, the Waters column still yielded sharper peaks than the completely porous 3 μ m C₁₈ column. In addition, separations on the Waters column were directly comparable to the Ascentis fused-core column. Due to these observations, and the fact that we have previously profiled all 192 rat mitochondria and serum samples using this RP LC-MS method, we choose to continue working with the Ascentis Express column. In the future, it may be advantageous to pursue a UPLC system,

in order to decrease the analysis time and increase sample throughput. In that case, a smaller particle size column would be the best choice.

HCD Fragmentation of Cis-Trans PG (18:1/18:1)

To determine if HCD fragmentation of the different isomers would yield diagnostic patterns that would be able to identify the double-bond stereochemistry in the absence of chromatographic separation, we ran an experiment where we alternated the MS full scans with 3 different HCD energies, 30, 60 and 100 eV. The four different scans were run consecutively in one experiment. The Ascentis chromatography yielded 6 second wide peaks, therefore, 3 total scan segments were acquired across each isomer. For both *cis* and *trans* PG (18:1/18:1), the preferential loss of the FA 18:1 side chains yielded identical patterns and isobaric fragments at all energies. Results can be found in the supplement for this manuscript. Figures S1a-d show the *cis* isomer, and S2a-d show the *trans* isomer. As expected, no differences were observed. Thus, the results from this experiment show that chromatographic separation is essential to distinguish PG *cis-trans* isomers.

Separation of Cis-Trans PG (18:1/18:1) in a Biological Matrix

Although we achieved baseline separation of the *cis-trans* isomers in neat solution, it was imperative to determine if the separation holds up in a matrix where multiple similar compounds could co-elute and potentially compromise the separation mechanism. To test this, we spiked a *cis-trans* mixture into a previously extracted serum pool sample. Figure 3 shows the total ion chromatogram (TIC) of this pool sample in panel A, with panel B representing the XIC of ion m/z 773.5333 as described previously in Figure 2. The separation in panel B clearly shows the *cis* and *trans* PG (18:1/18:1) isomers with the same baseline separation, $W_{1/2}$, and retention time as when injected neatly, indicating that even in the presence of a large pool of lipid species isomer separation could be achieved and monitored.

Determination of Endogenous PC (18:1/18:1) in Mitochondria and Serum

We now use this approach to study endogenous biological lipids. PG (18:1/18:1) *cis-trans* pairs were used to assess the efficient RP chromatographic separation of our LC-MS method, however, the amount of endogenous *trans* PG (18:1/18:1) found in the rat serum and mitochondria was below our systems limit of quantitation. PC (18:1/18:1) *cis-trans* pairs were first assessed, in the same manner as the PG (18:1/18:1) *cis-trans* isomers, as neat standards and when spiked into a matrix, determining that the RP-LC-MS could indeed separate and detect the stereoisomers. Due to their increased signal in both the rat mitochondria and sera analyses, we then chose to study endogenous PC (18:1/18:1) *cis-trans* pairs. LC-MS profiling of the 192 animals undergoing dietary macronutrient study has been done previously and in these studies, three isomers with an m/z 786.5992 representing the $[M+H]^+$ ions of PC (18:1/18:1) *cis*, PC (18:0/18:2) and PC (18:1/18:1) *trans* lipids were found at RTs 14.39, 14.71 and 15.06 minutes respectively. The *cis* and *trans* PC (18:1/18:1) peaks were characterized by spiking a serum sample with the *cis* and *trans* internal standards. Figure 4 shows how the endogenous *cis* and *trans* PC (18:1/18:1) peaks increase in intensity after spiking.

We next confirmed that any signal observed for the earliest eluting PC (36:2) peak is originating from the PC (18:1/18:1) *cis* stereoisomer. Other possible PC (36:2) stereoisomers that can potentially co-elute with this PC (18:1/18:1) *cis* compound include PC (16:0/20:2), PC (16:2/20:0), PC (16:1/20:1), PC (14:0/22:2), or PC (14:1/22:1). Since mixed acyl standards, e.g., PC (16:1/20:1), are not available for direct comparison, we have eliminated the possibility of these compounds complicating the analysis by showing, through HCD fragmentation and an elongated isocratic separation, that none of the other

possible fatty-acid peaks chromatographically align with either: (i) each other, which would indicate that they are produced from the same parent ion, or; (ii) with the XIC of the parent peak of interest (supplementary figures S3a–e). Furthermore, PC (18:0/18:2) was characterized via HCD fragmentation and chromatographic alignment of the parent m/z 786.5992 at 14.71 minutes with the fragments for FA 18:0 and FA 18:2 (data not shown).

Due to the nature of this profiling experiment, stable isotope labeled internal standards are not used and therefore only relative quantitation is achieved. Due to chromatographic overlap and the isobaric nature of the stereoisomers (Figure 4), the PC (18:0/18:2) peak introduces some inherent background signal to both the PC (18:1/18:1)-*cis* and PC (18:1/18:1)-*trans* frame analysis quantitation. We therefore took two approaches to estimate the maximal potential contamination of the PC (18:1/18:1) values: (i) we manually assessed the total signal from the beginning of the PC (18:1/18:1) *cis* peak to the end of the PC (18:1/18:1) *trans* peak and then determined the fraction of that quantitation that would lie from the end of the PC (18:1/18:1) *cis* peak to the beginning of the PC (18:1/18:1) *trans* peak, using the peak boundaries determined in spiked samples, as in Figure 4. This analysis showed that a minimum of 87% of the PC (18:0/18:2) signal lay outside the regions quantitated for the PC (18:1/18:1) *cis* peak and the PC (18:1/18:1) *trans* peak. As this analysis assumes that there is no PC (18:1/18:1) *cis* peak or PC (18:1/18:1) *trans* peak, it places an absolute maximum of 13% of the PC (18:0/18:2) signal as a contaminant, with ~67% coming from the portion of the chromatogram which includes the PC (18:1/18:1) *cis* peak. (ii) As an independent means of assessing potential contamination, we also took the 192 samples scored for the diet study by SIEVE. In these 192 rat sera samples, the PC (18:1/18:1) *cis* peak had a minimal value of 13% of the PC (18:0/18:2) with a median value of 28%. The PC (18:1/18:1) *trans* peak had a minimal value of 5% of the PC (18:0/18:2), a median value of 9%, and a 20th percentile cut-off value of 7.5%. This last value was chosen as a cut-off and defined as the baseline for normalization of the *trans* peak; returning to the manual quantitation number (i) of 13%, we then defined the baseline cut-off for the PC (18:1/18:1) *cis* peak at 5.5%. As noted above, these are believed to represent the maximal percent of the PC (18:1/18:1) peaks that are actually from the PC (18:0/18:2) signal. Thus, the values presented later in this report are considered to be under-estimates, particularly of the *trans* peak, but, most importantly, they should be considered as relative rather than absolute values. This means that with a different background subtraction the values may shift in value, but the trends we are observing will go unchanged. All figures and comparisons are performed using the background subtracted values.

The presence of both the *cis* and *trans* FA chain isomers in PC (18:1/18:1) appears to be related to both the animal's primary dietary fat source and glycemic index (Figure 5). We note that the relative levels of both the *cis* and *trans* isomers changes in one or more diets, suggesting that these values should be considered in addition to the ratio between them. For example, the HGI diets in mitochondria (Figure 5A and B) show large quantitative changes in both the *cis* and the *trans* isomers between the two dietary fat sources. The *cis* isomer increases in signal by ~50% between the *trans* and MUFA (*cis*) diet while the *trans* isomer decreased by approximately the same amount. This quantitation pattern appears to hold true, with a few exceptions, implying that when there is an increased dietary pool of the *cis* FA the PC (18:1/18:1) *cis* isomer increases in signal and with an increased dietary pool of the *trans* FA the PC (18:1/18:1) *trans* isomer increases in signal. In the sera samples we found very little PC (18:1/18:1) *trans* (Figure 5C), note the 10 times lower y axis, in animals fed most of the MUFA diets, however, the PC (18:1/18:1) *cis* isomer shows the same quantitation pattern as was observed in mitochondria regarding the increased dietary pool of FA 18:1 driving the increase in PC (18:1/18:1) *cis* found in sera. These data suggest that although both isomers can be monitored in each biological sample, only the mitochondria

cis:*trans* ratio can be analyzed due to the low levels of PC (18:1/18:1)-*trans* found in sera from animals maintained on some of the MUFA diets.

Figure 5, panel E shows 8 bar graphs representing the cis:*trans* ratio of PC (18:1/18:1) found in each of the MUFA (white bars) and *trans* (black bars) fat diets analyzed from mitochondria. We observed that the cis:*trans* isomer ratio is consistently larger in those diets containing predominantly MUFA or FA 18:1 cis (white bars) fatty acids, indicating and increased presence of the PC (18:1/18:1)-cis isomer in those diets. It should be noted that the converse relationship is true if one plots the *trans*:cis ratio, meaning the *trans*:cis ratio is higher in those diets supplemented with *trans* fat (data not shown). These data suggest that differential presence of these isomers in PC side-chains can be monitored using this RP LC-MS profiling and isomer ratio technique.

The dietary study analyzed herein contained 24 diets, with 6 fat groups, not just the 2 (MUFA and *trans*) discussed up until now. When the animals are not being supplemented with either 18:1 FA isomer as in the MUFA and *trans* diets, for example in the PUFA or SFA diets, there are still endogenous amounts of both the cis and *trans* isomers incorporated into PC. This observation can be explained by noting that in rats, the 18:1 fatty acid can be made in both the cis and *trans* isomer forms. The cis 18:1 isomer is a non-essential fatty acid that can be produced by normal fatty acid biosynthesis pathways. In sera, it can be speculated that the cis isomer may be the favored configuration, due to it having a much larger average signal than the *trans* isomer across all the diets studied.

Although unexpected from a nutritional perspective, the *trans* isomer is observed in PC phospholipids even when there has been no such fat available via the animal's diet. Research has shown that the endogenous *trans* isomer is most likely a result of isomerization induced by thiyl radicals (RS•) adding across the cis double bond followed by β -elimination of the thiyl adduct to the *trans* isomer.^{26–28} These data suggest that increased endogenous *trans* 18:1 may represent evidence of increased RS• activity. In accordance with this, in mitochondria, the signal for the two PC (18:1/18:1) isomers is more comparable, suggesting a greater presence of the *trans* isomer regardless of diet. This type of increase would be expected in a system, such as mitochondria, where normal cellular respiration produces reactive oxygen species byproducts which in turn may yield more endogenous environmental RS• induced cis-*trans* biotransformation.

Free Radical Induced Cis-Trans Biotransformation

Thus far in our analysis, we have only considered two of the three possible PC (18:1/18:1) stereoisomers that may exist biologically, the di-cis and di-*trans* isomers. It is possible for a third isomer containing one cis FA 18:1 chain and one *trans* FA 18:1 chain (PC (18:1/18:1) cis-*trans*) to be produced during biotransformation and therefore potentially be detected within our system. As discussed previously, it has been shown that thiyl radicals can produce cis-*trans* isomerization^{25, 29}. We therefore selectively generated these biotransformed products using a PC (18:1/18:1) cis standard and following the work of Chatgililoglu et. al.²⁹ we incubated this standard with the free radical inducing agent AIBN. Under these conditions, the RS• induced biotransformation of cis to *trans* was achieved using the carbon radical (C•), produced from AIBN, to react with PhSH yielding the corresponding RS•. Results from this experiment indicate that, in fact, the mixed-acyl PC (18:1/18:1) cis-*trans* isomer is generated *in vitro* as seen in Figure 6 panel A, which shows the baseline separation of all three species. Our profiling data, however, does not show this particular cis-*trans* mixed isomer being present in appreciable amounts *in vivo*, as seen in Figure 6 panels B and C. These panels show the XIC of m/z 830.5917 (PC (36:2)) aligned with the fragmentation spectra for m/z 281.2481 (FA (18:1)), generated from the analysis of a mitochondrial pool sample. Only the fragments for the PC (18:1/18:1) cis and

PC (18:1/18:1) *trans* species are discernible using this method, labeled at RT 14.6 and 15.4 minutes respectively. There is a small FA (18:1) fragment at RT 14.97 minutes, however, this fragment corresponds to a phosphatidylethanolamine lipid which also elutes at this time, under these chromatographic conditions. Additionally, it appears that the PC (18:1/18:1) *cis-trans* species co-elutes significantly with the PC (18:0/18:2) lipid which dominates any available signal. Therefore, using this profiling method, we are only able to monitor the PC (18:1/18:1) *cis* and PC (18:1/18:1) *trans* species, and the amount of mixed acyl PC (18:1/18:1) *cis-trans* cannot be determined.

The results of this report reveal and highlights two key findings: (i) The ability of non-targeted RP LC-MS lipidomics profiling to provide PC (18:1/18:1) *cis* and PC (18:1/18:1) *trans* stereoisomer information enhances its utility to study the possible implications each lipid species can have on pathophysiological outcomes such as mitochondrial dysfunction and disease risk. (ii) An important advantage of discovery-based non-targeted LC-MS profiling, in comparison to more targeted methods, is the ability to re-analyze experiments when neither the biological importance nor the overall lipidome complexity is completely understood. This benefit was exploited in this *cis-trans* study, where the ability to determine the stereochemistry around the double bond via RP separation was unknown until after RP LC-MS profiles were already acquired. Re-analysis of the data set provided new details into the modulation of fatty acyl side chains based on diet, whereas with a targeted analysis method, the data would have had to be reacquired in order to test the *cis-trans* separation hypothesis.

It has become apparent that changes in chromatography may enhance this *cis-trans* analysis, especially due to the inherent chromatographic overlap of the more highly abundant PC (18:0/18:2) peak with both stereoisomers. To characterize the PC (18:1/18:1)-*cis* peak we ran a 120 minute isocratic run at 43% B, which helped separate out these compounds and gave insight into how a more specific separation can enhance future studies (see supplement). We note that future studies can address these problems in several ways: (i) using different chromatographic conditions to better determine the values in the pool samples better, then back-calculating in the corrected values; (ii) manual quantitation of all peaks so as to best minimize the overlap (vs SIEVE) and (iii) spiking internal standards particularly labeled for determining the percentage of each peak that overlaps. For this study, however, we focused primarily on re-analysis of profiling data allows for new and unique lipids to be studied as they are discovered and standards become available.

CONCLUSION

To our knowledge, this is the first data that describes the separation of phospholipid *cis-trans* isomers both in and out of a matrix using a RP LC-MS approach. The significance of this work can be applied to various other biologically important lipids containing FA chains which can vary by the stereochemistry around a double bond. Currently, this method is limited by the availability of authentic standards that can be used to determine chromatographic elution patterns due to the *cis-trans* isomers being completely isobaric, yielding identical fragmentation by HCD. As these standards become available, however, this method will ideally be applied to other lipid classes, such as glycerolipids more specifically triacylglycerides, where the presence of *trans* fatty acyl side-chains may provide enhanced knowledge regarding individual disease risk and overall health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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– PG (18:1/18:1) – Cis

– PG (18:1/18:1) - Trans

Figure 1.

PG (18:1/18:1) cis-trans isomers, showing how the structure changes due to the stereochemistry around each double bond.

Figure 2.

The PG (18:1/18:1) cis and *trans* separation for the Ascentis Express, Waters and Phalanx columns respectively. Each panel represents the XIC of m/z 773.5333 representing the $[M-H]^-$ ion of the standards.

Figure 3.

Separation of *cis-trans* PG (18:1/18:1) isomers in a serum pool sample. Top panel shows the TIC of the serum pool and the bottom panel is the XIC of m/z 773.5338 representing the [M-H]⁻ ion of the PG (18:1/18:1) isomers that were spiked into the pool sample.

Figure 4.

Two XICs of m/z 786.5992 representing the $[M+H]^+$ ion of PC (36:2). Panel A is a serum pool sample, and panel B shows that same sample spiked with the PC (18:1/18:1) *cis* and *trans* isomers. Peaks at 14.32 and 15.06 minutes represent the *Cis* and *Trans* isomer respectively.

Figure 5.

panels A–D represents the average signal of PC (18:1/18:1) cis and PC (18:1/18:1) *trans* in mitochondria (A–B) and serum (C–D). Panel E shows the cis-*trans* ratio of PC (18:1/18:1) found in mitochondria from rats fed each of the MUFA (white bars) and *Trans* (black bars) fat diets. The two high glycemic index diet pairs (HGI) are followed by the medium high glycemic index diets (MHGI), the medium low (MLGI) and finally the low glycemic index diets (LGI). The bars represent the average signal for each molecule, with background subtraction, across all rats in the diet (n=8), and error bars represent the standard error of the mean. Statistical significance was assessed via t-tests without correction for multiple comparisons.

Figure 6.

panel A shows the results from the PC (18:1/18:1) *cis-trans* biotransformation experiment and the baseline separation of all three species, the PC (18:1/18:1) *cis*, PC (18:1/18:1) *cis-trans* and PC (18:1/18:1) *trans*. Panels B and C show how in the mitochondrial pool analysis, only the PC (18:1/18:1) *cis* and PC (18:1/18:1) *trans* species can be accounted for, due to co-elution of the PC (18:1/18:1) *cis-trans* with the PC (18:0/18:2) isomer.