

Characterization of complex lipid mixtures in contaminant exposed JEG-3 cells using liquid chromatography and high-resolution mass spectrometry

Eva Gorrochategui · Josefina Casas ·
Elisabet Pérez-Albaladejo · Olga Jáuregui ·
Cinta Porte · Sílvia Lacorte

Received: 4 October 2013 / Accepted: 6 June 2014 / Published online: 28 June 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract The aim of this study was to develop a method based on ultra high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) for lipid profiling in human placental choriocarcinoma (JEG-3) cells. Lipids were solid–liquid extracted from JEG-3 cells using a solution of chloroform/methanol (2:1, v/v) in a simple procedure requiring minimal sample alteration. Simultaneous separation of complex lipid mixtures in their major classes was achieved with a reversed-phase (C_8) UHPLC column and a mobile phase containing methanol with 1 mM ammonium formate and 0.2 % formic acid (A)/water with 2 mM ammonium formate and 0.2 % formic acid (B). Lipids were characterized using time-of-flight (TOF) and Orbitrap under full scan and positive electrospray ionization mode with both analyzers. A total of 178 species of lipids, including 37 phosphatidylcholines (PC), 32 plasmalogen PC, 9 lyso PC, 4 lyso plasmalogen PC, 30 triacylglycerols, 22 diacylglycerols, 7

cholesterol esters, 25 phosphatidylethanolamines, and 12 sphingomyelins, were identified using TOF and Orbitrap. The identification of all lipid classes was based on exact mass characterization with an error < 5 ppm. The developed methodology was applied to study lipid alterations in human placental cells against the exposure to perfluorinated chemicals (PFCs) and tributyltin (TBT).

Keywords Lipidomics · JEG-3 cells · Mass spectrometry · Time-of-flight and orbitrap analyzers · Perfluorinated chemicals (PFCs) · Tributyltin (TBT)

Introduction

Lipidomics, a ramification of metabolomics, is the end point of omics cascade and can be described as the system wide study of lipids and their interaction with other biochemicals. In fact, the term lipidome can be defined as the comprehensive and nonexhaustive quantitative description of a group of lipid classes that may constitute a cell or bio-organism (Castro-Perez et al. 2010). Lipids and their interaction with cells play a crucial role in living organisms. Among the multiple biological functions of lipids, they contribute in compartmentalization, energy production and storage, cell-signaling processes, protein trafficking, and membrane organizing tasks (Oresic et al. 2008; Van Meer 2005). Moreover, several diseases, including obesity (Shi and Burn 2004), cardiovascular dysfunctions, diabetes, cancer, and neurodegenerative alterations, are associated with abnormalities in lipid functions and physiological levels (Shui et al. 2007). Lipid alterations have been attributed to the possible involvement of environmental obesogens, xenobiotics that can disrupt the normal developmental and homeostatic control over adipogenesis and energy balance (Grün and Blumberg 2006). Among others, tributyltin (TBT) has raised a lot of

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-014-3172-5) contains supplementary material, which is available to authorized users.

E. Gorrochategui · E. Pérez-Albaladejo · C. Porte · S. Lacorte (✉)
Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA), Consejo Superior de Investigaciones Científicas (CSIC), Jordi Girona 18-26, Barcelona 08034, Catalonia, Spain
e-mail: slbqam@cid.csic.es

J. Casas
Department of Biomedical Chemistry, Institute of Advanced Chemistry of Catalonia (IQAC), Jordi Girona 18-26, Barcelona 08034, Catalonia, Spain

O. Jáuregui
Scientific and Technological Centers, University of Barcelona, Baldiri Reixac, 10-12, Barcelona 08028, Catalonia, Spain

attention since it is an environmental endocrine disrupter with the capacity to promote adipogenesis (Grün et al. 2006). In addition to TBT, perfluorinated chemicals (PFCs) have been reported to alter lipid levels in some animal species and humans (Gilliland and Mandel 1996; Nelson et al. 2010). Both TBT and PFCs share bioaccumulative properties; they are widely distributed environmental pollutants and are the target analytes of the present study.

According to all that, the importance of lipidomics lays not only on its contribution toward the enhanced understanding of the pathogenesis of multiple disease states related to lipids but also on the study of the impact of some emerging chemicals such as obesogen compounds in the environment and humans.

Lipids are exceptionally diverse in their structural, chemical, and physical properties. According to the latest version of the Comprehensive Classification System for Lipids firstly established on 2005 by the International Lipid Classification and Nomenclature Committee (ILCNC), lipids are divided into eight main distinctive classes: (1) fatty acids (FA), (2) glycerolipids (GL), (3) glycerophospholipids (GP), (4) sphingolipids (SP), (5) sterol lipids (ST), (6) prenol lipids (PR), (7) saccharolipids (SL), and (8) polyketides (PK) (Fahy et al. 2009). The characterized lipids in the present study include some GL (diacylglycerols and triacylglycerols), various GP (phosphatidylcholines and its lyso forms and derivatives with plasmalogens and phosphatidylethanolamines), sphingomyelins in the group of SP, and cholesterol esters as ST.

Lipid extraction is the first step toward lipidomic analysis. Due to the water-insoluble nature of lipid molecules, most of the described procedures in literature use organic solvents as the preferred extractive agents. Among the organic solvents, the most common for lipid extraction in biological tissues are chloroform and methanol combined in a mixture (2:1, v/v; Bligh and Dyer 1959; Folch et al. 1957). However, nontraditional extractive methods have used other organic solvents, such as isopropanol or hexane, in order to maximize the selective collection of particular lipid classes of interest (Hughes and Brash 1991).

Separation of the extracted lipid species in their major classes is a complicated procedure due to the highly complex biological matrices in which they are contained. One of the earliest techniques developed was thin layer chromatography (TLC) for routine analysis of lipids (Bennett and Heftmann 1962; Ruggieri 1962). However, in most recent applications, this technique is only used as a fast and extensive screening tool prior to the exhaustive analysis with more sensitive and selective techniques such as liquid chromatography (LC; Watson 2006).

Lipidomic analysis by LC can follow either normal-phase or reverse-phase strategies. While fatty acids are commonly separated on reverse-phase columns (Watson 2006), separation of phospholipids (PL) can be achieved by both

approaches. Normal-phase LC effectively separates PL according to their different polar heads and not considering their sn-1 and sn-2 fatty acid substituent. In contrast, reversed-phase strategy separates PL on the basis of their fatty acid residues (Castro-Perez et al. 2010). However, for a complete separation of lipids, two-dimensional LC has reported to be the ideal method since it allows the combination of normal- and reversed-phase approaches (Pulfer and Murphy 2003; Wang et al. 2013).

Various classic detection methods, such as spectrophotometric analysis in the ultraviolet (UV) range and evaporative light scattering, have reported to be adequate for detecting lipids (Watson 2006). However, in recent years, mass spectrometry (MS) has evolved as a superior detection method for identifying lipids in biological matrices due to its high sensitivity and the additional information it provides (Sommer et al. 2006). Moreover, the recent ability of high-resolution mass spectrometers to obtain accurate mass measurements has replaced them at the top of MS analyzers in lipidomic research. In fact, analyzers, such as Orbitraps, Fourier transform ion cyclotron resonance (FTICR), time-of-flight (TOF), and hybrid quadrupole orthogonal TOF (Q-TOF), have replaced the conventional low-resolution quadrupoles and linear ion traps, as they can resolve isomeric and isobaric species and elucidate elemental composition.

Thus, the aim of this study was to characterize the lipidomic composition of JEG-3 cells using high-resolution mass spectrometry (HRMS). Moreover, the effects of PFCs and TBT on the lipidome of the human placental choriocarcinoma cell line JEG-3 were investigated.

Materials and methods

Chemicals and reagents

Minimum essential medium, fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin G, streptomycin, phosphate buffered saline (PBS), and trypsin-ethylenediaminetetraacetic acid (EDTA) were supplied by Gibco BRL Life Technologies (Paisley, Scotland, UK). Tributyltin (TBT), perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA), and perfluorohexanesulfonate (PFHxS) were purchased from Sigma-Aldrich (Steinheim, Germany), and perfluorobutanesulfonate (PFBS) and perfluorooctanesulfonate (PFOS) were obtained from Fluka (Austria). Stock standard solutions containing the mixture of the eight PFCs were prepared in ethanol at concentrations of 0.1 and 1 mM, and the stock solution of TBT was prepared in dimethyl sulfoxide (DMSO) at a concentration of 0.02 mM. These solutions were stored at -20°C . High-performance liquid chromatography (HPLC)-

grade water, methanol (>99.8 %), and acetonitrile (>99.8 %) were purchased from Merck (Darmstadt, Germany). Chloroform was supplied by Carlo-Erba (Peypin, France) and butylated hydroxytoluene (BHT) by Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

JEG-3 cells derived from a human placental carcinoma were obtained from American Type Culture Collection (ATCC HTB-36). They were grown in Eagle's minimum essential medium supplemented with 5 % fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate, and 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified incubator with 5 % CO₂ at 37 °C. Cells were routinely cultured in 75 cm² polystyrene flasks (Corning; NY, USA). When 90 % confluence was reached, cells were dissociated with 0.25 % (w/v) trypsin and 0.9 mM EDTA (trypsin-EDTA) for subculturing and experiments. Experiments were carried out on confluent cell monolayers.

Sample preparation

Cell exposure to PFCs and TBT

Cells were seeded at a rate of 0.67×10^6 cells per well (six-well plate) and allowed to attach overnight in an incubator at 37 °C, 5 % CO₂. Then, 6 µl of the 0.02 mM stock solution of TBT or 6 µl of the 0.1 and 1 mM stock solutions containing the eight PFCs of study (PFBA, PFHxA, PFOA, PFNA, PFDoA, PFBS, PFHxS, and PFOS) were directly added to the wells. The final concentration of DMSO and ethanol in culture wells was 0.4 % (v/v), and final concentrations of PFCs were 0.6 and 6.0 µM and 0.1 µM for TBT. After 24 h of exposure, the medium was aspirated and cells were washed with PBS, trypsinized, and centrifuged at $5,400 \times g$ for 10 min. The supernatant was aspirated and cells were stored at -80 °C until analysis. In all cases, addition of the tested compound was done in triplicate, and controls were performed by adding the corresponding solvent to cells.

Lipid extraction

Lipids were extracted from JEG-3 cells with similar extraction conditions of a previous study (Christie 1985). To the cell pellets, 540 µl of a methanol:chloroform (1:2, v/v) solution containing 0.01 % of BHT, acting as an antioxidant, were added. Samples were shaken with a vortex mixer (1 min), were settled for 30 min, and extracted in an ultrasonic bath for 5 min at room temperature (two times). Between each period of 5 min, samples were thoroughly mixed. Afterwards, samples were centrifuged at $13,000 \times g$ for 5 min. The supernatant was transferred to a new micro vial, evaporated to dryness,

reconstituted with 160 µl of acetonitrile, and stored at -20 °C in an argon atmosphere.

Instrumental analysis

UHPLC conditions

All analyses were performed with an ultra high performance liquid chromatography (UHPLC) system using an octyl carbon chain (C₈)-bonded silica column. Chromatographic parameters, such as column temperature, injection volume, flow rate, mobile phases, and gradient elution programs, are summarized in Table 1.

MS conditions

Analyses were performed with an UHPLC system coupled to two distinct mass analyzers. The analytical instrumentation used were an UHPLC system coupled to a Waters/LCT Premier XE TOF analyzer controlled with Waters/Micromass MassLynx 4.1 software and an UHPLC system (Accela) coupled to a Thermo Fischer Scientific LTQ Orbitrap Velos controlled with Thermo Fischer Scientific/Xcalibur software. The MS parameters, such as the ionization mode (positive electrospray), the mass acquisition range used in each mass analyzer along with other parameters, are summarized in Table 1.

Identification and relative quantification of lipids

Positive identification of lipids was based on the accurate mass measurement with an error < 5 ppm using both high-resolution TOF and Orbitrap. Relative retention times in UHPLC, compared to that of some standards (± 2) used in a previous study, analyzed under the same chromatographic conditions (Garanto et al. 2013), were also considered as identification criteria. An inventory of a total of 225 lipids, containing 45 phosphatidylcholines (PC), 36 plasmalogen PC, 18 lyso PC, 4 lyso plasmalogen PC, 39 triacylglycerols (TAG), 26 diacylglycerols (DAG), 7 cholesterol esters (CE), 35 phosphatidylethanolamines (PE), and 15 sphingomyelins (SM), based on reported identified species (Garanto et al. 2013), was first generated. Their theoretical exact masses were determined using a spectrum simulation tool of Xcalibur software, and the obtained list was further used as a home-made referential database. Individual chromatographic peaks of distinct lipid species were isolated from full-scan MS spectra when selecting their theoretical exact masses, extracted from the database. Then, a list of possible candidates fitting the specific exact mass was generated using formula determination tools (elemental composition search) of both Micromass MassLynx and Thermo Fischer Scientific Xcalibur softwares. The elemental number was restricted to include C, H, O, N, and P. The formula constraints were C, H,

Table 1 UHPLC and MS conditions tested for the analysis of lipids in JEG-3 cells

Conditions	A	B
<i>UHPLC</i>		
LC system	Waters ACQUITY UHPLC system	Thermo Fischer Scientific Accela UHPLC system
Column	ACQUITY UPLC BEH (Waters, Ireland) C ₈ column (100×2.1 mm) 1.7 μm	Then a list of possible candidates fitting
Column T	30 °C	30 °C
Injection volumen	10 μl	5 μl
Flow rate	0.3 ml/min	
Mobile phase	A: Methanol with 1 mM ammonium formate and 0.2 % formic acid	B: Water with 2 mM ammonium formate and 0.2 % formic acid
Gradient elution	80–90 % of A/3 min, held for 3 min, increase to 99 % A/9 min, held for 3 min, return to initial conditions in 2 min and stabilization for 3 min	85 % of A/1 min, increase to 90 % A/9 min, held for 2 min, increase to 99 % A in 6 min, held for 2 min, return to initial conditions in 2 min and stabilization for 3 min
<i>MS</i>		
MS system	Waters/ LCT Premier XE time-of-flight (TOF) analyzer	Thermo Fischer Scientific Orbitrap analyzer
Ionization mode	ESI (+)	ESI (+)
Acquisition range	50–1,800 <i>m/z</i>	395–1,000 <i>m/z</i>
Capillary voltage	3.0 kV	Source voltage 3.5 kV
	Desolvation temperature 350 °C	Capillary temperature 300 °C
	Desolvation gas flow 600 l/h	Sheath gas flow 50 l/h
		Auxiliar gas flow 20 l/h
		Sweep gas flow 2 l/h

$O \geq 1$, $P \geq 0$, and $N \geq 1$, following the nitrogen rule. The number of double-bond equivalents (DBEs) was set between -0.5 and 15.0 . The search was based on single mass analysis and only considered the *m/z* value of the monoisotopic peak.

Annotation of lipid species: GL, GP SP, and ST are annotated as <lipid subclass> <total fatty acyl chain length>:<total number of unsaturated bonds> and SM <total fatty acyl chain length>:<total number of unsaturated bonds in the acyl chain>. Relative quantification was done by comparison of peak areas in extracted ion chromatograms between exposed cells and controls.

Data processing and statistical analysis

Significant differences among controls and TBT/PFC-exposure treatment mean values ($n=3$) were determined by an ANOVA of one factor, considering statistical significance of $P < 0.05$. All graphics were computed in Excel 2007.

Results and discussion

Chromatographic separation

Successful UHPLC separation of lipids in their major classes was achieved using a C₈-bonded silica column of 100-mm

length. With TOF, 10 μl of the sample extract provided good resolution and sensitivity. However, with the Orbitrap, improved chromatographic peak shape was obtained when injecting 5 μl instead of 10 μl into the system and optimum column temperature was set to 30 °C. Concerning mobile phases, the optimum conditions were the use of methanol with 1 mM ammonium formate and 0.2 % formic acid (A) combined with water with 2 mM ammonium formate and 0.2 % formic acid (B). The addition of ammonium formate to the solvents has reported to be advantageous due to the formation of adduct ions $[M+NH_4]^+$ in positive mode, which are known to be more stable than the $[M+H]^+$ ions for some lipid classes. In the present study, lipids corresponding to the families of TAG, DAG, and CE were identified as ammonium adducts, whereas the rest of lipid groups were identified in the monoprotonated form. However, the use of ammonium formate requires the addition of buffering acids, such as the formic acid added in the present study, to avoid the formation of double-peak chromatograms (Sommer et al. 2006). Regarding elution program, the gradient conditions slightly changed when using the TOF or the Orbitrap with the aim to improve and adapt the chromatographic separation to each instrument. Both gradients presented in conditions A and B of Table 1 allowed good separation of main lipid classes. They both started at high percentage of the organic phase A, 80 and 85 %, respectively, and increased up to 99 % to allow the

desired elution of lipid species. With the Orbitrap, the two gradients were tested, and enhanced separation of lipid species was achieved when using the latter conditions, despite having a longer chromatogram than the one used with TOF. Figure 1 shows UHPLC chromatograms of TOF (Fig. 1a) and Orbitrap (Fig. 1b) when working at their respective optimum conditions (Table 1).

MS conditions

As observed in Table 1, the main difference among MS parameters used with TOF and Orbitrap was the acquisition range. The shorter range used in Orbitrap with respect to the one used in TOF was necessary to suppress high contribution of background ions in the system.

Lipid identification

Lipid species were successfully identified with both high-resolution TOF and Orbitrap. As shown in the

UHPLC chromatograms of Fig. 1a and b, equivalent chromatographic distribution profiles were obtained when using both TOF and Orbitrap. Lyso PC and lyso plasmalogen PC were the first groups of lipids to elute and appeared in the early 5 min of the UHPLC-TOF chromatogram and between 4 and 7 min of the UHPLC-Orbitrap chromatogram. These groups of lipids were totally resolved from PC, plasmalogen PC, PE, SM, and DAG, which eluted together in the subsequent 10 min of the chromatogram. TAG and CE appeared together in the final minutes of the chromatogram. Individual lipid species unresolved in the total ion chromatogram were successfully isolated when their exact masses were selected. Thus, despite the incomplete chromatographic resolution of the complex lipid mixture, identification of individual lipids was possible using both the high-resolution TOF and Orbitrap. A total of 178 species of lipids were identified by TOF and Orbitrap and are shown in Table 2. In the group of glycerophospholipids, 107 species were identified containing 37 PC, 32 plasmalogen PC, 9 lyso PC, 4 lyso plasmalogen PC, and 25 PE. In the group of

Fig. 1 UHPLC-MS total ion chromatograms of extracted lipids from JEG-3 cells when using **a** the TOF analyzer and **b** the Orbitrap analyzer, under conditions A and B of Table 1, respectively

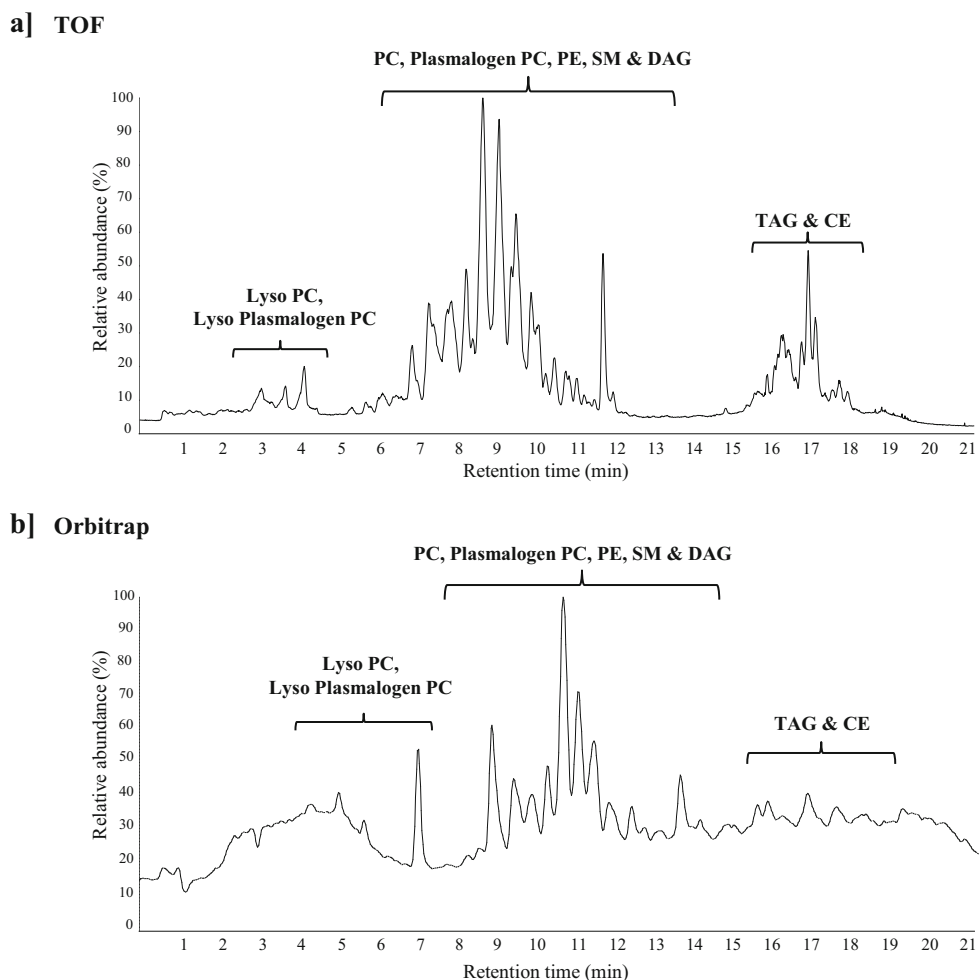


Table 2 Lipid species identified using both TOF and Orbitrap analyzers

<i>Glycerophospholipids</i>	
PC	30:0 ^{##} , 32:0, 32:1, 32:2, 34:0, 34:1, 34:2, 34:3, 34:4, 34:5 ^{##} , 34:6 [#] , 36:0, 36:1, 36:2, 36:3, 36:4, 36:5, 36:6, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6 ^{##} , 38:8 ^{##} , 40:0, 40:1, 40:2, 40:3, 40:4, 40:5, 40:6, 42:2, 42:3, 42:4, 42:5, 42:6
Plasmalogen PC	30:0, 30:1, 30:2 ^{##} , 32:0, 32:1, 32:2, 34:0, 34:1, 34:2, 34:3, 34:4 ^{##} , 36:0, 36:1, 36:2, 36:3, 36:4, 36:5, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6, 38:7, 40:1, 40:2, 40:3, 40:4, 40:5, 40:6, 40:7, 40:8 ^{##}
Lyso PC	16:0, 16:1, 18:0, 18:1, 18:2, 20:1, 20:2, 20:3, 20:4
Lyso plasmalogen PC	16:0, 16:1 ^{##} , 18:0, 18:1
PE	32:0, 32:1, 34:0, 34:1, 34:2, 34:3 [#] , 36:0 ^{##} , 36:1, 36:2, 36:3, 36:4, 36:6 ^{##} , 38:1, 38:2, 38:3, 38:4, 38:5, 38:6, 38:7 [#] , 40:1, 40:2 [#] , 40:3, 40:4 ^{##} , 40:5 ^{##} , 40:6 ^{##}
<i>Glycerolipids</i>	
TAG	48:0, 48:1, 48:2, 48:3, 48:4, 48:6 ^{##} , 50:0, 50:1, 50:2, 50:3, 50:4, 50:5, 50:6, 52:0, 52:1, 52:2, 52:3, 52:4, 52:5, 52:6, 52:7, 54:1 [#] , 54:2, 54:3, 54:4, 54:5, 54:6, 54:7, 54:8, 54:9
DAG	32:0, 32:1, 32:2, 32:3, 34:0, 34:1, 34:2, 34:3, 34:4, 36:0, 36:1, 36:2, 36:3, 36:4, 36:5, 36:6, 38:0 ^{##} , 38:1 [#] , 38:2, 38:3, 38:4, 38:5
<i>Sterol lipids</i>	
CE	18:0, 18:1, 18:2, 18:3, 20:4, 20:5, 22:6
<i>Sphingolipids</i>	
SM	14:0, 16:0, 16:1, 18:0, 18:1, 20:0, 20:1, 22:0, 24:0, 24:1, 24:2, 24:3

Symbols (#) indicate lipid species only identified using TOF and (##) using Orbitrap. Lipid species GL, GP SP, and ST are annotated as <total fatty acyl chain length>:<total number of unsaturated bonds> and SM as <total fatty acyl chain length>:<total number of unsaturated bonds in the acyl chain>

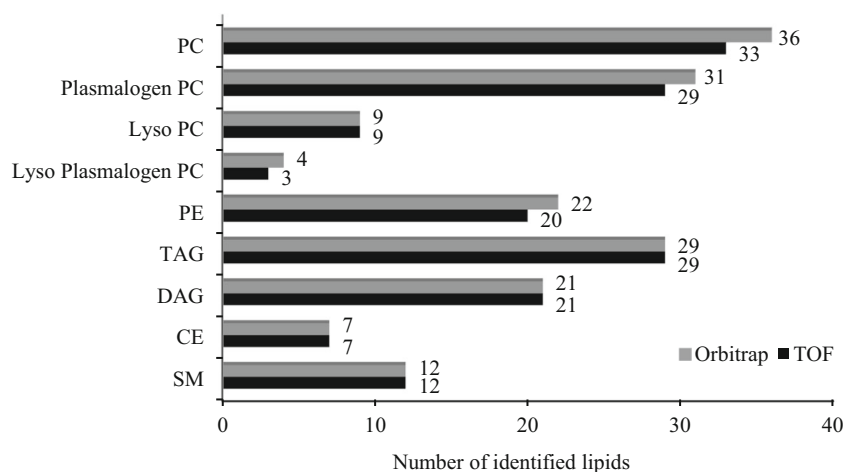
glycerolipids, 52 species were identified containing 30 TAG and 22 DAG. As sterols, 7 CE were identified,

and in the group of sphingolipids, 12 SM were determined. The compounds identified with their accurate mass measurement, elemental composition, calculated mass, error, double-bond equivalents and retention times are shown in the Table S1. Although not all lipids present in the cells were analyzed, the reported classes are quite representative since account for the 70 % of cellular lipidome. Phosphatidylserine and phosphatidylinositol were not determined since they can only be detected in negative electrospray ionization mode and the sensitivity was too low. considering the amount of extracted lipids from JEG-3 cells.

The distinct resolution of TOF and Orbitrap, 11,500 and 30,000 FWHM at *m/z* 556 and 400, respectively, resulted in a high lipid identification power. The use of 30,000 resolution with the Orbitrap would allow the resolution of isobaric species, although in our specific case, such species were chromatographically resolved. Figure 2 shows the identification ratio of the different target lipids when using both mass spectrometers. Equal results were obtained in families of lyso PC, TAG, DAG, CE, and SM. In the rest of the lipid groups, PC, plasmalogen PC, lyso plasmalogen PC and PE, identification was slightly superior with Orbitrap but with no significant differences. Of all the 178 lipids identified in the present study, 88 % were found by both analyzers, 4 % were only seen by TOF, and 8 % were only found with Orbitrap. Thus, the two high-performance platforms, TOF and Orbitrap analyzers, showed equivalent capability for lipidomic analysis in human placental choriocarcinoma JEG-3 cells.

Lipidome changes in PFC/TBT-exposed cells

The chromatographic profiles of PFC/TBT-exposed cells were compared to controls. TBT effects were studied at the low concentration of 0.1 μ M, since it was the reported level at

Fig. 2 Number of identified lipid species when using TOF and Orbitrap analyzers

which this compound promoted adipogenesis in vertebrates (Grün et al. 2006). In contrast, PFC effects were studied at two levels of concentration, 0.6 and 6 μM , the first close to the TBT exposure level and the latter about ten times higher. The selected doses of exposure

were nontoxic for JEG-3 cells, as shown in a previous study (Gorrochategui et al. 2014).

Figure 3 represents differences in lipid amounts of exposed cells and controls caused by the distinct treatments, expressed as increasing rates, calculated as:

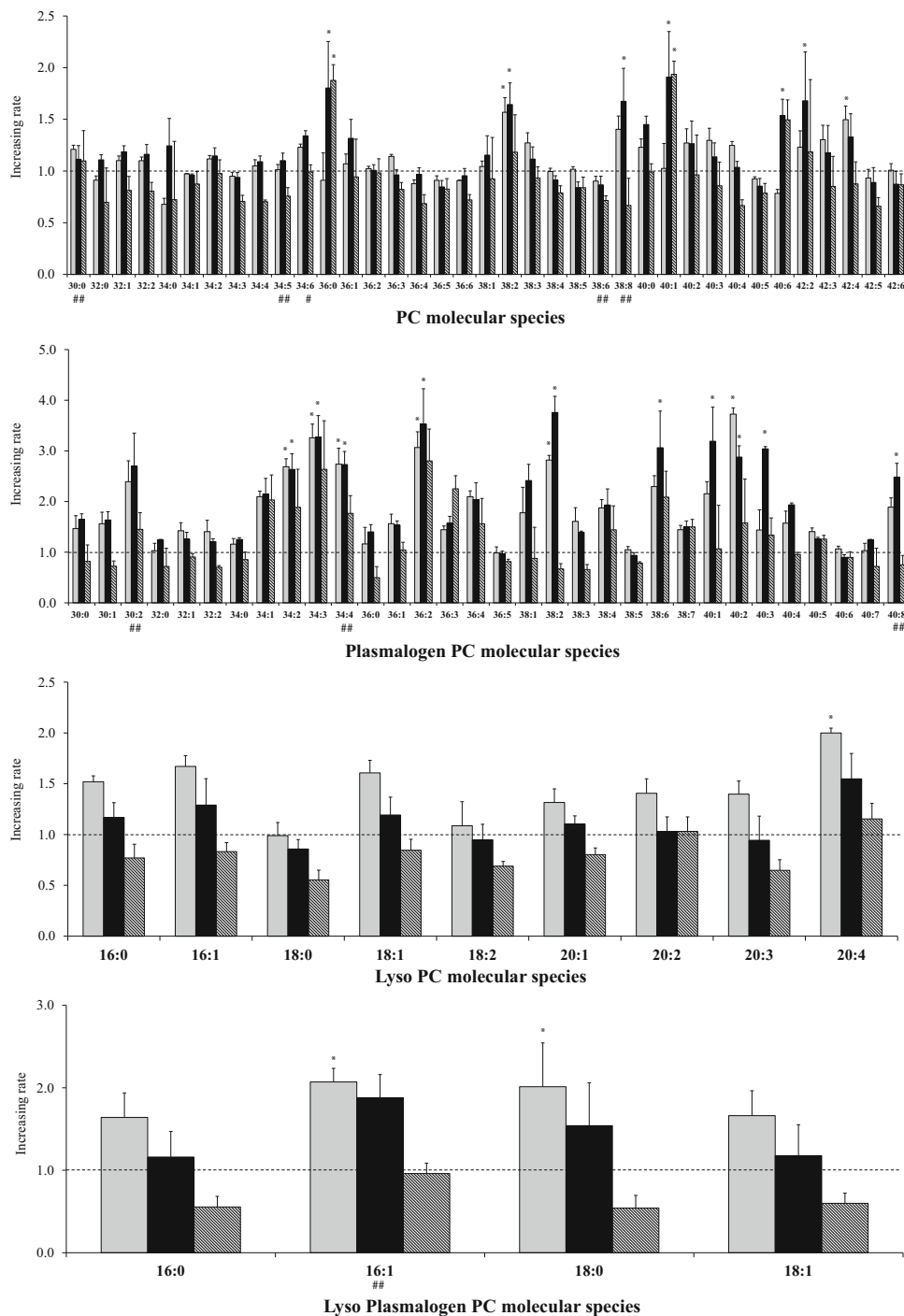


Fig. 3 Increasing rates of **a** PC, plasmalogen PC, lyso PC and lyso plasmalogen PC, **b** TAG, DAG and CE and **c** PE and SM in JEG-3 cells exposed to a mixture of PFCs, at 0.6 μM (□) and 6 μM (■) and TBT at 0.1 μM (▨). Values are relative to the cell control (set to 1) and are means \pm SEM ($n=3$), analyzed by TOF. Symbols (#) indicate lipid species

only identified using TOF and (##) using Orbitrap. One-way ANOVA was performed to indicate statistical significant differences against the control ($*P < 0.05$). Species of lipids are defined by their number of carbon atoms and the unsaturations of their fatty acid chains

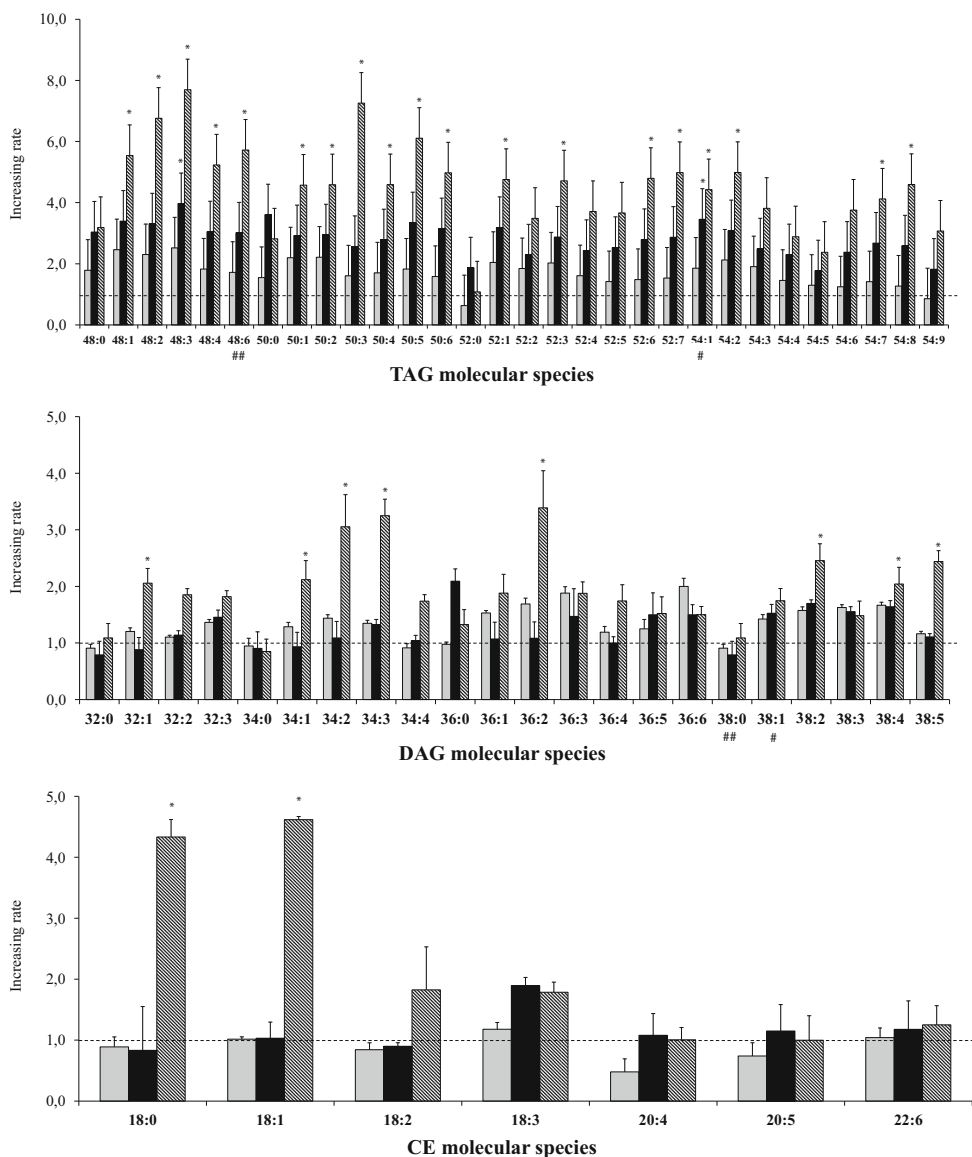


Fig. 3 (continued)

$$\text{Increasing rate} = \frac{\sum_{i=1}^n A_i, \text{exposed cell}}{\sum_{i=1}^n A_i, \text{control}}$$

where A_i represents the peak area of the lipid, and n the number of replicates of the four groups of samples (controls, cells exposed to PFCs at 0.6 μM , PFCs at 6 μM , and TBT), which was three in our experiment conditions. In each case, the variance associated to the increasing rate was calculated as the quotient of the standard error of the mean (SEM) of the corresponding cell treatment and SEM of controls.

Despite most lipid species were identified using both analyzers, the results shown correspond to the TOF analysis with the exception of few lipid species only identified with Orbitrap. Lipid species exclusively identified with TOF and Orbitrap are highlighted with symbols # and ##, respectively.

As shown in Fig. 3a, distinct effects were observed in four groups of the GP analyzed, PC, plasmalogen PC, lyso PC, and lyso plasmalogen PC, when exposed to PFCs and TBT. While exposure to PFCs produced significant increase in some lipid species, there was no significant alteration resulting from TBT exposure in the majority of cases. Even more, in groups of lyso PC and lyso plasmalogen PC, levels of lipids of cells exposed to the

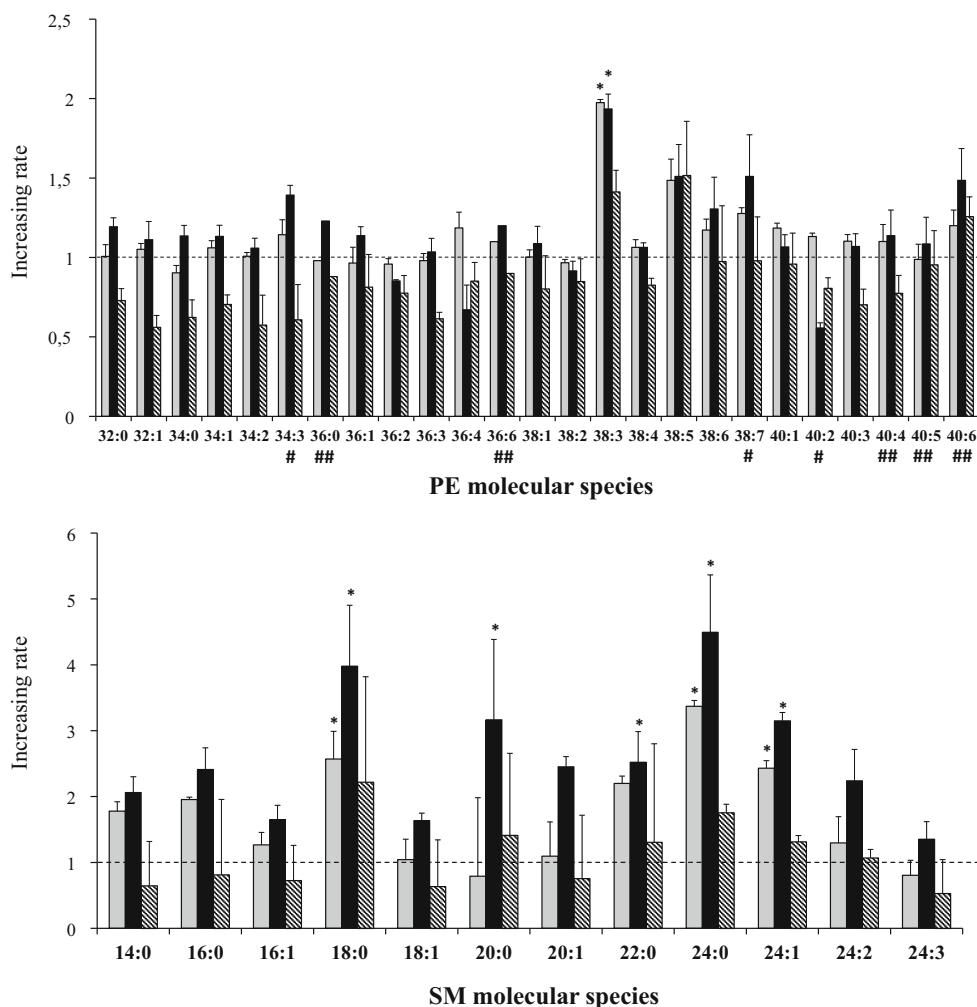


Fig. 3 (continued)

organotin compound were lower than in control samples. Surprisingly, effects of TBT were highly visible in families of TAG and DAG, whose levels suffered a dramatic increase after the exposure to the organotin compound. The effect of PFCs was considerably minor, even in the high concentration exposure of 6 μ M, producing a low increase in the amount of some TAG and no significant effect on DAG. In the case of CE species, only two of them, 18:0 and 18:1, suffered a significant increase when exposed to TBT. Finally, effects on PE were practically undetectable and were only noticed in lipid species 38:3, whereas effects on SM resulted higher and were attributed to the presence of PFCs.

The observed increase in the amount of lipids resulting from the presence of TBT is in accordance to the findings of several studies which report the obesogenic effects of the

organotin compound. According to our findings, Janer et al. (2007) reported increased accumulation of lipids and fatty acids in ramshorn snail *Marisa cornuarietis*. Moreover, Grün et al. (2006) reported promoted adipogenesis in murine 3 T3-L1 cell model and elevated lipid accumulation in adipose depots, liver and testis of neonate mice exposed to TBT. The observed effects of PFCs suggested a major influence on membrane lipids containing phosphorylcholine, such as PC, plasmalogen PC, lyso PC, and lyso plasmalogen PC, and sphingomyelins. In contrast, alterations of TBT were highly noticeable in TAG, DAG, and CE species.

In conclusion, the presented UHPLC-TOF/Orbitrap approaches allowed successful lipid profiling of JEG-3 cells. The proposed methodology was applied for the study of lipid alterations in PFC/TBT-exposed cells observing significant effects of both xenobiotics.

Acknowledgments This study was financed by the INNPACTO project (IPT-2011-0709-060000). Dr. R. Chaler, D. Fanjul, and Eva Dalmau are acknowledged for TOF-MS support and Dr. Alberto Adeva for Orbitrap-MS support.

References

- Bennett RD, Heftmann E (1962) Thin-layer chromatography of sterols. *J Chromatogr A* 9:359–362
- Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Phys* 37:911–917
- Castro-Perez JM, Kamphorst J, Degroot J, Lafeber F, Goshawk J, Yu K, Shockcor JP, Vreeken RJ, Hankemeier T (2010) Comprehensive LC-MSE lipidomic analysis using a shotgun approach and its application to biomarker detection and identification in osteoarthritis patients. *J Proteome Res* 9:2377–2389
- Christie WW (1985) Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J Lipid Res* 26:507–512
- Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CRH, Shimizu T, Spener F, Van Meer G, Wakelam MJO, Dennis EA (2009) Update of the LIPID MAPS comprehensive classification system for lipids. *J Lipid Res* 50:S9–S14
- Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497–509
- Garanto A, Mandal NA, Egado-Gabás M, Marfany G, Fabriàs G, Anderson RE, Casas J, González-Duarte R (2013) Specific sphingolipid content decrease in Cerkl knockdown mouse retinas. *Exp Eye Res* 110:96–106
- Gilliland FD, Mandel JS (1996) Serum perfluorooctanoic acid and hepatic enzymes, lipoproteins, and cholesterol: a study of occupationally exposed men. *Am J Ind Med* 29:560–568
- Gorrochategui E, Pérez-Albaladejo E, Casas J, Lacorte S, Porte C (2014) Perfluorinated chemicals: differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells. *Toxicol Appl Pharmacol* 277(2):124–130. doi:10.1016/j.taap.2014.03.012
- Grün F, Blumberg B (2006) Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinol* 147: S50–S55
- Grün F, Watanabe H, Zamanian Z, Maeda L, Arima K, Cubacha R, Gardiner DM, Kanno J, Iguchi T, Blumberg B (2006) Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. *Mol Endocrinol* 20:2141–2155
- Hughes MA, Brash AR (1991) Investigation of the mechanism of biosynthesis of 8-hydroxyeicosatetraenoic acid in mouse skin. *BBA-Lipid Lipid. Met* 1081:347–354
- Janer G, Navarro JC, Porte C (2007) Exposure to TBT increases accumulation of lipids and alters fatty acid homeostasis in the ramshorn snail *Marisa cornuarietis*. *Comp Biochem Phys C* 146:368–374
- Nelson JW, Hatch EE, Webster TF (2010) Exposure to polyfluoroalkyl chemicals and cholesterol, body weight, and insulin resistance in the general U.S. population. *Environ Health Persp* 118:197–202
- Oresic M, Hänninen VA, Vidal-Puig A (2008) Lipidomics: a new window to biomedical frontiers. *Trends Biotechnol* 26:647–652
- Pulfer M, Murphy RC (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev* 22:332–364
- Ruggieri S (1962) Separation of the methyl esters of fatty acids by thin layer chromatography. *Nature* 193:1282–1283
- Shi Y, Burn P (2004) Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. *Nat Rev Drug Discov* 3: 695–710
- Shui G, Bendt AK, Pethe K, Dick T, Wenk MR (2007) Sensitive profiling of chemically diverse bioactive lipids. *J Lipid Res* 48:1976–1984
- Sommer U, Herscovitz H, Welty FK, Costello CE (2006) LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures. *J Lipid Res* 47:804–814
- Van Meer G (2005) Cellular lipidomics. *EMBO J* 24:3159–3165
- Wang S, Li J, Shi X, Qiao L, Lu X, Xu G (2013) A novel stop-flow two-dimensional liquid chromatography-mass spectrometry method for lipid analysis. *J Chromatogr A* 1321:65–72
- Watson AD (2006) Thematic review series: systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. *J Lipid Res* 47: 2101–2111