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RPLC-Ion-Trap-FTMS Method for Lipid Profiling of Plasma: Method Validation and Application to p53 Mutant Mouse Model

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A reversed-phase liquid chromatography-linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometric method was developed for the profiling of lipids in human and mouse plasma. With the use of a fused-core C_8 column and a binary gradient, more than 160 lipids belonging to eight different classes were detected in a single LC-MS run. The method was fully validated and the analytical characteristics such as linearity (R^2, 0.994−1.000), limit of detection (0.08−1.28 μg/mL plasma), repeatability (RSD, 2.7−7.9%) and intermediate precision (RSD, 2.7−15.6%) were satisfactory. The method was successfully applied to p53 mutant mice plasma for studying some phenotypic effects of p53 expression.

Keywords: Lipidomics • Lipid Profiling • Metabolite Profiling • Metabolomics • FTMS • p53 Mouse Model

1. Introduction

Lipids comprise an enormous number of chemically distinct molecular species arising from the various combinations of fatty acids and a series of backbone structures.[1] Lipids are broadly defined as hydrophobic or amphoteric small molecules. The vital role of lipids in pathophysiology is substantiated by a large number of studies on human diseases as a consequence of disruption of lipid metabolic enzymes and pathways.[1−3] For example, lipids contribute to common pathophysiological states such as fatty liver and lipotoxic induced insulin resistance, diabetes, Alzheimer's disease, atherosclerosis, and toxic manifestations of infectious diseases.[1,4] In addition, the role of lipids in the formation of cell membranes promotes them to be both possibly a ligand and substrate for proteins,[5] so that lipid data can complement proteomics data.[6] Despite its importance, lipid profiling research (or lipidomics), and metabolome research in general, was somewhat overshadowed by the explosive progress in genomics and proteomics over the past decade. The complexity of lipids and the resulting analytical challenges are certainly playing a role in this. Therefore, a powerful method for lipid profiling analysis has become increasingly important.

During the last decades, a large variety of methods for lipid analysis have been published, either based on thin-layer chromatography or based on high-performance liquid chromatography (HPLC) equipped with ultraviolet or evaporative light-scattering detection.[7−11] Because of the great variety in lipid structure and polarity, the biological samples were usually prefractionationed in lipid classes prior to further separation. However, such an approach cannot fulfill high-throughput analysis and cannot provide the required structural information of the lipids due to the limitations of the detector. Gas chromatography−mass spectrometry (GC-MS) is an alternative, and still plays an important role in lipid analysis. However, GC-MS-based lipid profiling methods need usually time-consuming procedures for hydrolysis and derivatization, often applied after prefractionation of lipid fractions.[12] Without hydrolysis and derivatization, many of the lipid classes are not GC amenable. Still, hydrolysis and derivatization may hamper quantification. In recent years, LC-MS has become an increasingly popular approach for lipid analysis.[13−15] Among the methods of ionization commonly used in LC-MS, electrospray ionization (ESI) and atmospheric pressure chemical ionization are most popular. LC-ESI-MS allows a rapid and sensitive analysis of lipids, which offers possibilities for improved determination of minor compounds and potential biomarkers. Normal-phase HPLC coupled with MS (NPLC-MS) was extensively used for analysis of lipids in cells and tissues and body fluids, as well as in plants.[16−22] However, in most cases, long analysis time, poor peak shapes and limited resolution were the main shortcomings of these methods.[20] Reversed-phase HPLC-MS (RPLC-MS) has the advantage of compatibility of the mobile phase of the

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HPLC with biological and lipophilic samples and is widely used for profiling, identification and quantification of lipids in serum, plasma, bacteria and tissue samples.[23−29] It was noticed that the shortcomings encountered with NPLC-MS were not all overcome in RPLC-MS, but selectivity and sensitivity were generally better. Different approaches for lipid analysis have been reviewed in detail.[1,6]

To our knowledge, so far the reports on extensive method validation for lipid profiling analysis by RPLC-MS in complex biological matrices are limited.[23] A validation of a profiling method should comprise in our opinion at least of the characterization of linearity, limit of detection (LOD), repeatability, intermediate precision and, preferably also, recovery, as is proposed by the ICH[30] and IUPAC guidelines.[31] However, in most cases, only part of a full method validation was considered.[7,9,10,24] Barroso and Bischoff[26] developed a RPLC-MS method for phospholipids (PLs) and lyso-PLs analysis in human bronchoalveolar lavage fluid and reported a relatively complete method validation. They assessed the linear range of four PLs standards of glycerophosphocholine (GPCho), glycerophosphethanolamine (GPEtn), glycerophosphglycerol (GPGro) and monoacyl-glycerophosphcholine (lyso-GPCho) dissolved in phosphate buffered saline solution and also used sample spiking. Unfortunately, no data were reported on between-day variation. Furthermore, the chromatographic separation was less good and there was obviously a problem of peak tailing. Laaksonen and colleagues[23] developed a UPLC-Tof-ESI-MS method for lipid analysis in serum and tissue samples, which was extensively validated. Some minor elements of validation, as the between-day variation, were not reported. For some unknown reasons, the linearity determined from serum was suboptimal for lipid standards of GPCho (17:0/0:0), GPCho (17:0/17:0) and TG (17:0/17:0/17:0) at the concentration range of 10−250 μg/mL.

Recently, FTMS has been increasingly utilized in the profiling of lipids[14,32−36] due to its unparalleled mass accuracy and high mass resolution. In the present study, a sensitive method is described for the profiling of lipids in human and mouse plasma. The method is based on RPLC coupled with linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometric detection using electrospray ionization (RPLC-ESI-LTQ-FTMS). The chromatography was based on fused-core small diameter (2.7 μm) particles, consisting of a thin porous shell (0.5 μm thickness) of high-purity silica and modified with C_8 chains surrounding a solid silica core. These particles should enable higher column efficiencies compared with the conventional LC columns due to the shallow diffusion paths in the 0.5 μm thick porous shells and the small overall particle size. The developed method optimized the peak shapes of all lipid classes, and subsequently was fully validated in terms of linearity, LOD, repeatability and recovery.

The potential of the lipid profiling method was applied to the analysis of plasma samples from p53 mutant mice. P53 is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. These p53 mice have been engineered to carry a mutation in their p53 gene. The mutant mice carried the modified p53 gene in either one allele of the chromosome or in both alleles of the chromosome (heterozygous or homozygous selectivity). The aim of this application was to study phenotypic effects of p53 expression and mutations at the metabolite level using lipid profiling.

2. Experimental Procedures

2.1. Chemicals. ULC-MS grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and HPLC grade dichloromethane (DCM) were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium formate (AmFm, 99.995%) was purchased from Sigma (St. Louis, MO). Lipid standards of 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine GPCho (17:0/0:0), 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine GPCho (19:0/0:0), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine GPEtn (15:0/15:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine GPEtn (17:0/17:0), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) GPGro (14:0/14:0), 1,2-diheptadecanoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) GPGro (17:0/17:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine GPCho (17:0/17:0) and 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine GPCho (19:0/19:0) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2,3-Tripentadecanoylglycerol TG (15:0/15:0/15:0) and 1,2,3-triheptadecanoylglycerol TG (17:0/17:0/17:0) as well as cholesteryl heptadecanoate ChoE (17:0) were obtained from Sigma (Zwijndrecht, The Netherlands).

2.2. Lipid Nomenclature. Lipid nomenclature was carried out according to Lipid Maps (http://www.lipidmaps.org) throughout the article. For example, lyso-glycerophosphocholine with 17:0 fatty acid chain was named as monoacylglycerophosphocholine GPCho (17:0/0:0) or lyso-GPCho (17:0) and 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine was named as GPCho (19:0/19:0). In case of the fatty acid chain was not determined, the sum of the carbon number and double bond number of all fatty acyl side chains was given, for example, GPCho (32:0).

2.3. Preparation of Standards. Lipid stock solutions were prepared by weighing an exact amount of the lipid standard in a DCM/MeOH mixture (2:1, v/v) resulting in a concentration of about 2 mg/mL and were stored at −20 °C. For analysis, the stock solutions were diluted to appropriate concentrations with a DCM/MeOH mixture (2:1, v/v). The mixture of internal standards (IS) consisted of GPCho (17:0/0:0) (15 μg/mL), GPGro (17:0/17:0) (20 μg/mL), GPEtn (17:0/17:0) (20 μg/mL), GPCho (17:0/17:0) (40 μg/mL) and TG (17:0/17:0/17:0) (25 μg/mL) in a DCM/MeOH mixture (2:1, v/v). A second mixture of standards ("validation standard mixture") was typically used for method validation and consisted of GPCho (19:0/0:0), GPGro (14:0/14:0), GPEtn (15:0/15:0), GPCho (19:0/19:0) and TG (15:0/15:0/15:0) for different compounds at different concentration ranges in a DCM/MeOH mixture (2:1, v/v), for example, the solution of intermediate concentration contained GPCho (19:0/0:0) at 15 μg/mL, GPGro (14:0/14:0) at 20 μg/mL, GPEtn (15:0/15:0) at 15 μg/mL, GPCho (19:0/19:0) at 40 μg/mL, TG (15:0/15:0/15:0) at 25 μg/mL.

2.4. Extractions. A total lipid fraction was obtained by liquid−liquid extraction (LLE) of 30 μL of human or mouse plasma with a DCM/MeOH mixture (2:1, v/v), according to the method of Bligh and Dyer[37] with some slight modification.

2.4.1. For Method Application. Briefly, 30 μL of IS were added to 30 μL of human plasma sample followed by addition of 190 μL of MeOH, then 380 μL of DCM was added and the mixture was vortexed for 20 s both before and after adding DCM. Subsequently, 120 μL of water was added and vortexed for 10 s to form a two-phase system. The lower organic phase contained virtually all of the lipids, while the upper aqueous phase contained much of the nonlipid compounds. After

equilibration for 10 min at room temperature, the extract was centrifuged at 8000*g* for 10 min at 10 °C. A total of 340 μL of lipid extract from the organic phase was collected and then diluted 5 times with injection solvent (ACN/IPA/H_2O = 65:30:5, v/v/v) prior to analysis. Ten microliters was injected into the LC-MS system.

2.4.2. For Method Validation: Spiking before Sample Preparation. Thirty microliters of IS and 30 μL of the validation standard mixture were added to 30 μL of human plasma sample followed by addition of 180 μL of MeOH and then 360 μL of DCM. The same procedures as described in section 2.4.1 were subsequently performed till centrifugation. A total of 340 μL of lipid extract from the lower organic phase was collected and then 60 μL of 2:1 DCM−MeOH was added. This mixture was further diluted 5 times with injection solvent; 10 μL was injected into the LC-MS system.

2.4.3. For Method Validation: Spiking after Sample Preparation. The same procedures as those described in section 2.4.2 were performed except 60 μL of 2:1 DCM−MeOH instead of 30 μL of IS plus 30 μL of validation standard mixture was added to 30 μL of human plasma before sample preparation and 30 μL of IS plus 30 μL of validation standard mixture instead of 60 μL of 2:1 DCM−MeOH were added to the extract after sample preparation. For the blank sample, 30 μL of human plasma was replaced by 30 μL of HPLC-MS grade water and the 60 μL of the two sets of standard mixtures were replaced by 60 μL of 2:1 DCM−MeOH.

2.5. HPLC Analysis. A Surveyor LC system with autosampler and MS pump (Thermo Fischer) was used for this study. Samples of lipid extracts were injected on an Ascentis Express C_8 2.1 × 150 mm (2.7 μm particle size) column (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). This column was packed with the fused-core small diameter (2.7 μm) particle, consisting of a 1.7 μm solid core and a 0.5 μm porous shell of high-purity silica. These particles have a major benefit of small diffusion path (0.5 μm) compared to conventional fully porous particles and are able to achieve high speed and high efficiencies but at much lower backpressures. A binary gradient system of ACN−water (60:40, v/v) and IPA−ACN (90:10, v/v), both containing 10 mM AmFm, was used as eluent A and B, respectively. The elution was performed with a gradient of 30 min; during 0−1.5 min isocratic elution with 32% B; from 1.5 to 4 min increase to 45% B, from 4 to 5 min increase to 52% B, from 5 to 8 min to 58% B, from 8 to 11 min to 66% B, from 11 to 14 min to 70% B, from 14 to 18 min to 75% B, from 18 to 21 min to 97% B, during 21 to 25 min 97% B is maintained; from 25−25.1 min solvent B was decreased to 32% and then maintained for another 4.9 min for column re-equilibration. The flow-rate was 0.26 mL/min. The column oven temperature was maintained at 55 °C and the temperature of the autosampler tray was set to 12 °C.

2.6. Mass Spectrometry. The HPLC system was coupled online with a hybrid linear ion trap-Fourier-transform ion cyclotron resonance mass spectrometer (Thermo Fisher, San Jose, CA) equipped with an ESI source (ESI-LTQ-FTMS). The effluent from the HPLC column entered the MS through a stainless steel ES ionization needle with an internal i.d. of 50 μm, and the spray voltage was set at 3.8 kV in positive ion mode. The heated capillary was set at 300 °C. The sheath gas flow was 60 units and the auxiliary gas flow was 5 units. The capillary voltage offset was 28.5 eV in positive ion mode. The ion source and ion optic parameters were optimized with respect to the protonated molecular ion of GPCho (17:0/17:0) during direct infusion experiments. Data acquisition of LC-MS analysis occurred using both FTMS in high resolution and ion trap. MS^n experiments in the ion trap were carried out with relative collision energy of 30−40% and the trapping of product ions were carried out with a *q*-value of 0.18, and the product ions were analyzed in the ion trap. Maximum injection time was 200 ms and number of μscans was 2. The full scan range was 400−1300 *m*/*z*. The data were collected in centroid mode. For investigation, other lipid internal standards, that is, GPGro (17:0/17:0), GPEtn (17:0/17:0), and TG (17:0/17:0/17:0) as well as standard of ChoE (17:0) were used for tuning rather than GPCho (17:0/17:0), and the influence of the obtained tune files on the analysis of lipid species was studied.

2.7. Application to Plasma of p53 Mutant Mice. The lipid profiling LC-MS method was applied to 12 mice plasma samples from p53 mutant mice with different gender and genotypes which were kindly provided by Ludwig Institute for Cancer Research[38] (United Kingdom). Among those, 6 samples were from male mice and 6 from female mice; 6 from heterozygous mice and 6 from mice homozygous for the modified p53 gene. The quality control sample (QC) was prepared by pooling and mixing 20 μL from each sample. Each sample was extracted in duplicate and spiked with the five internal standards before extraction and each extract was analyzed once by RPLC-LTQ-FTMS ($n = 2$ per sample). As QC samples, 6 aliquots of the pooled QC were each extracted once and each extract analyzed in duplicate. In total, 36 samples were analyzed in randomized order.

2.8. Data Processing. LCquan v2.5 (Thermo Fisher) was used for the determination of the peak areas of the internal standards and 92 selected lipid species, which were present in all of the samples, from the smoothed extracted ion chromatograms acquired with the LTQ using a mass window of 1 Da, as the present version of the quantitation software could not handle the high resolution data. The parameters for the integration were peak detection algorithm, ICIS; smoothing points, 7; window, 30 s; view width, 3 min; baseline, 40; area noise factor, 5; peak noise factor, 10. Prior to data analysis, all lipid peaks were normalized. The lipids were normalized using the internal standards as follows: monoacyl-glycerophosphocholines (lyso-GPChos), monoacyl-glycerophosphoethanolamines (lyso-GPEtns) and sphingomyelins (SMs) were normalized with GPCho (17:0/0:0); glycerophosphoethanolamines (GPEtns) and diglycerides (DGs) were normalized with GPEtn (17:0/17:0); glycerophosphocholines (GPChos) was normalized with GPCho (17:0/17:0); and cholesterol esters (ChoEs) as well as triglycerides (TGs) were normalized with TG (17:0/17:0/17:0). Statistical data analysis and data visualization were carried out using SIMCA-P (version 11.0.0.0) software from Umetrics (Umea, Sweden) and Microsoft Office Excel 2003 (Microsoft, Redmond, WA).

3. Results and Discussion

3.1. HPLC-MS Analysis. The initial experiments were focused on optimizing the separation of the lipid extract using an Alltima HP C_8 2.1 × 150 mm (5 μm particle size) column performed at 25 °C with a ternary gradient of A (10% ACN−90% water, 10 mM AmFm), B (45% ACN−55% IPA, 10 mM AmFm) and C (50% IPA−50% DCM) at a flow rate of 0.30 mL/min. This method showed a baseline separation among lyso-GPChos, PLs and TGs, but somewhat tailing peaks were obtained for GPChos and the separation of TGs was not satisfactory (data not shown). Though the separation time for the analytes was less

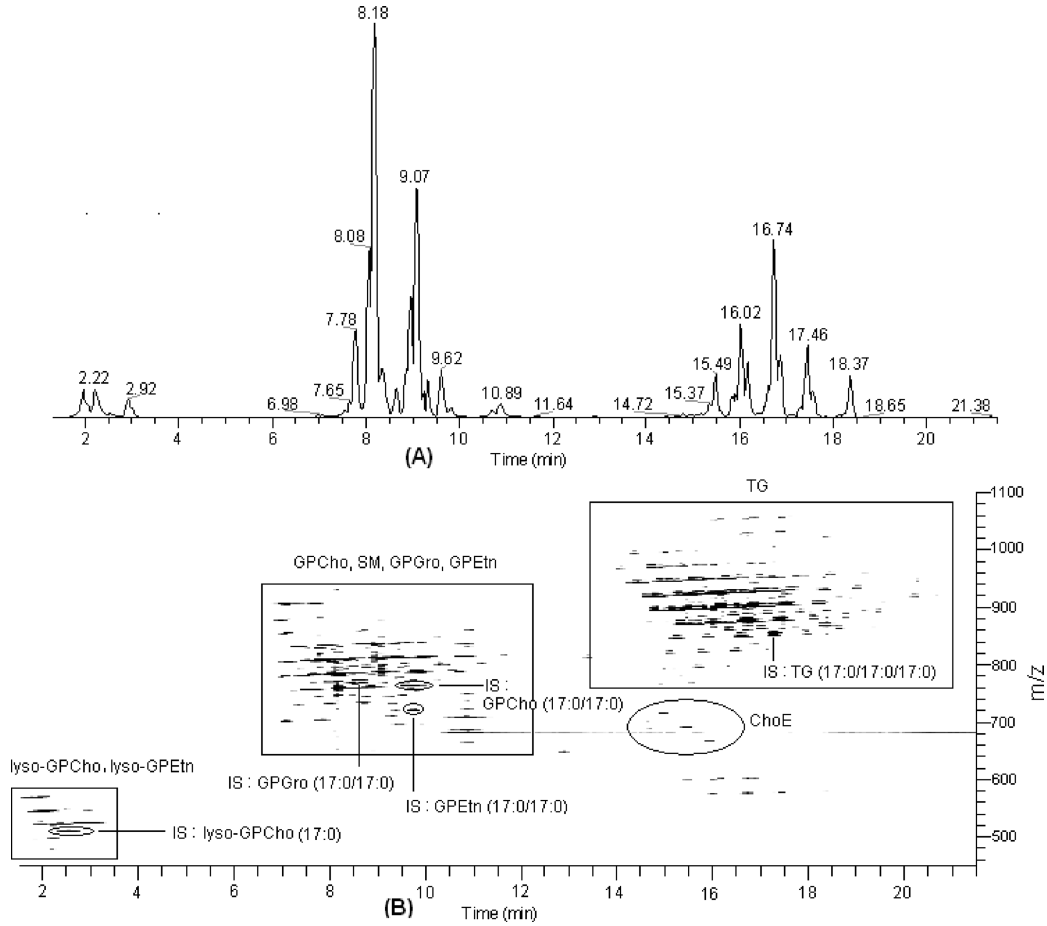

Figure 1. (A) RPLC-ESI-LTQ-FTMS chromatogram (base peak of LTQ) of 30 μL of mouse plasma after LLE sample preparation as described in Experimental Procedures, performed with a binary gradient of solvent A (40% water−60% ACN, 10 mM AmFm) and solvent B (10% ACN−90% IPA, 10 mM AmFm). (B) Density map (*m/z* vs retention time) of a part of the same chromatogram.

than 30 min, the total analysis time was 1 h due to prolonged system re-equilibration necessary because of the poor compatibility of DCM and water in the same system. In addition, it appeared not to be necessary to elute the most apolar lipids with DCM and the final gradient proved not to result in any significant carryover of lipids from one run to the next run (see section 3.3). After some optimization, finally, an Ascentis Express C_8 column (2.1 × 150 mm, 2.7 μm particle size) with a binary gradient of solvent A (60% ACN−40% water, 10 mM AmFm) and solvent B (90% IPA−10% ACN, 10 mM AmFm) was used. With this system, a better chromatographic performance, that is, sharper peaks of PLs without tailing, and, in addition, a 50% reduced analysis time were obtained, compared to the Allitima column with the DCM gradient as mentioned above. The HPLC packing was so-called Fused Core particles, which comprise a thin porous shell of high-purity silica surrounding a solid silica core. This fused core particles offer still a reasonable surface area of about 150 m^2/g with an average pore size of 90 Å useful for the analysis of metabolites, and an increased efficiency due to the shallow diffusion paths in the 0.5 μm thick porous shell and small overall particle size of 2.7 μm at a conventional pressure. A flow rate of 260 μL/min and a column temperature of 55 °C were chosen to keep the required pressure below 400 bar despite the relative high viscosity of IPA in solvent B. A typical LC-ESI-LTQ-FTMS chromatogram of mouse plasma is shown in Figure 1A. It can be seen from the density map (Figure 1B) that the group of the PLs was baseline separated from the group of the weak polar lipid species such as TGs and ChoEs. Lipid species from eight lipid classes of lyso-GPChos, lyso-GPEtns, SMs, GPEtns, DGs, GPChos, ChoEs and TGs were detected in positive ion mode. The separation of PLs and TGs in our method was improved as well as the peak shape compared with some of the methods reported before.[8,23,26,29] This became obvious from the comparison of the base peak chromatograms, in which the peaks were more spread over the chromatogram in our method than those in reference with even shorter analytical time, and from comparison of the peak shapes and the resolution of selected typical extracted ion chromatograms. Extracted ion chromatograms of RPLC-ESI-LTQ-FTMS of several typical lipid species in mouse plasma are shown in Figure S1 (see Supporting Information). Lyso-GPChos, lyso-GPEtns, SMs, GPEtns and GPChos were detected as $[M + H]^+$, while DGs, ChoEs and TGs were detected as $[M + NH_4]^+$. In addition, for most of the lipids, a minor $[M + Na]^+$ signal with relative intensity of less than 2% was found. In total, more than 160 lipid species were detected. It was noted that in the separation of lyso-GPChos the main peaks showed a shoulder of a coeluting peak having exactly the same mass (isomers). This may be caused by the different position of the fatty acid chain in sn-1 and sn-2 of the glycerol group. The same phenomenon was reported by Barroso and Bischoff.[26] For quantitative analysis, the peak areas of both isomers were summed.

To optimize detection, the tune parameters were optimized for five different classes (GPCho, GPEtn, GPGro, TG and ChoE). It was found that the main differences in optimized tuning

parameters between the different lipid classes were the values of sheath gas flow, capillary and tube lens offset. Out of these three parameters, the value of tube lens offset played the most important role. An extracted mouse plasma lipid sample analyzed with the five different tune parameter settings was obtained. It was found that the tune parameters optimized for GPCho, GPEtn, GPGro and TG yielded similar results, which was evaluated by the absolute peak areas of the randomly selected peaks from different lipid species. Finally, the tune parameters optimized for GPCho were overall slightly better than the others for all lipids tested. The tune parameters optimized for ChoE improved the signal of ChoEs several times and slightly improved the intensity of TGs, but it was not satisfactory for most of the other lipids (data not shown). Therefore, the tune parameters optimized for GPCho were used for the current lipid analysis method. The final method was validated by characterization of the linearity, repeatability, intermediate precision, recovery and LOD.

3.1.1. Linearity and LOD. For the developed RPLC-MS method, the linearity was determined for five different nonendogenous lipids of the validation standard mixture spiked to 30 μL of human plasma sample at six different concentrations (C0−C6) (see Table S1 of the Supporting Information). Different concentration ranges for linearity were used for different lipid classes by considering the concentrations of different lipid classes in the plasma samples. The mean peak area of four or six replicate measurements ($n = 4$ or 6) at each concentration level was calculated from the smoothed ion extracted chromatograms as described in the Experimental Procedures. For each lipid standard, a calibration curve was constructed using the mean of the ratios of the peak area of the lipid and its corresponding IS (both types of standards were spiked before extraction) of four or six replicate measurements (see Table S1 in Supporting Information). The linear regression coefficients R^2 were found to be better than 0.994 for all five nonendogenous lipids. The slopes, intercept and R^2 of the calibration lines as well as the average of the peak area ratios and relative standard deviation (RSD) of the peak area ratios are given in Table S1 in Supporting Information. Comparable results for the linearity and RSDs at the different concentration levels were obtained for the five nonendogenous lipids when using for all lipids GPCho (17:0/17:0) as internal standard (data not shown). The LODs were calculated as the minimum injected amount which gave a response in the MS detector as high as 3 times of signal-to-noise (S/N) ratio by using the corresponding smoothed ion extracted chromatograms. The LODs were satisfactory with values of 0.010 ng for lyso-GPCho, 0.171 ng for GPGro, 0.050 ng for GPEtn, 0.033 ng for GPCho and 0.036 ng for TG, or 0.08−1.28 μg/mL plasma, respectively (Table 1).

3.1.2. Repeatability. The repeatability was assessed by performing repeated sample preparation and analysis on a human plasma sample during three consecutive days (each day 3 sample was extracted and analyzed in duplicate). Briefly, one human plasma sample was divided into several aliquots which were stored at −20 °C. Each day 1 aliquot was used for the repeatability testing. Eleven lipids belonging to four different lipid classes in human plasma were randomly selected to determine the repeatability. Selection was only based on their abundance level and the retention time, and three lyso-GPChos, GPChos and TGs at low, middle and high abundance and two GPEtns at low and middle abundance were selected (for their estimated concentration, see Table S2 in the Supporting Information). The within-day repeatability and between-day variation were calculated as the mean ratios of the peak area of the selected lipid species from human plasma sample and the peak area of the corresponding IS spiked to the plasma sample prior extraction. The RSDs of between-day variation were calculated using ANOVA. The within-day RSD ranged from 3 to 8% with an average RSD of 5%. The between-day RSD ranged from 3 to 16% with the average RSD of 8% (Figure 2). The relatively highest between-day variation was found for TGs, but an RSD of 16% or less was considered acceptable for lipid profiling.

Table 1. Summary of Validation of RPLC-LTQ-FTMS of the Five Nonendogenous Lipids of the Validation Mixture Spiked Plasma Prior to Sample Preparation

lipid	linearity[a] R^2	limit of detection (μg/mL plasma)	repeatability[b] (RSD, %)	recovery[c] (%)
GPCho (19:0/0:0)	1	0.08	4.5	83
GPGro (14:0/14:0)	0.999	1.28	1.3	69
GPEtn (15:0/15:0)	1	0.4	2.5	96
GPCho (19:0/19:0)	0.994	0.26	3.2	98
TG (15:0/15:0/15:0)	0.998	0.29	3.6	101

[a] The calibration line was constructed using the mean ratio of the peak area of the lipid and the corresponding IS (see Experimental Procedures). [b] The repeatability is given for the second lowest concentration level (0.3−0.8 μg/mL) except for GPGro (14:0/14:0) at the level of 4 μg/mL because of its less good limit of detection. [c] Average recovery for three different plasma samples is given (for more details, see Figure 3 and text). For additional information such as concentration of different concentration levels, see Table S1 in the Supporting Information.

3.1.3. Recoveries. The recovery experiment was carried out for the five nonendogenous lipids of five different lipid classes of the validation mixture; these lipids were spiked before and after extraction to three different plasma samples, that is, two different human plasma samples and one plasma sample from mice carrying a mutant p53 at an intermediate concentration level (see Experimental Procedures). Triplicate sample preparations were performed for each plasma sample and each pretreated sample was analyzed by RPLC-ESI-LTQ-FTMS in duplicate (6 replicates per plasma sample in total). Recoveries were calculated for each lipid as the ratio of the peak area of that lipid in the sample spiked prior to extraction and in the samples spiked after sample preparation. The recoveries of each of the five lipids were comparable for the three different plasma samples (Figure 3). For GPEtn, GPCho and TG, the recovery obtained from mouse plasma was a little lower than those from human plasma samples which might be due to some matrix effect. The recoveries were 80−85% for the lyso-GPCho lipid, 67−71% for the GPGro lipid, 90−100% for the GPEtn lipid, 91−105% for the GPCho lipid and 94−105% for the TG lipid. The related RSDs ($n = 6$) of the recoveries per sample were 2−4% for lyso-GPCho, 2−5% for GPGro, 2−5% for GPEtn, 1−3% for GPCho and 2−4% for TG. Both, recoveries and RSDs were considered acceptable for sample analysis from complex biological matrices. As the linearity determined by addition of nonendogenous lipids to the plasma sample prior to sample extraction was linear (Table 1), and the recovery was satisfactory, it can be concluded that also the recoveries and performance at low lipid concentrations are satisfactory. This can be also deduced from the rather constant ratio of the peak area ratio of the lipids spiked prior to sample preparation and the concentration level at which they were spiked (using the data

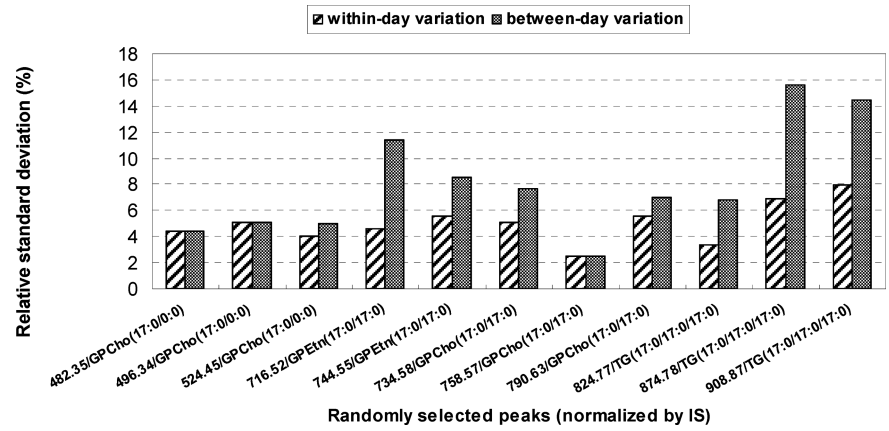

Figure 2. Within-day and between-day RSDs of randomly selected lipids in the sample (peak areas were normalized to the corresponding IS). The repeatability was assessed by performing sample preparation in triplicate and analysis in duplicate of human plasma samples at three consecutive days. The lipids were randomly selected, taking lipids of varying abundance (low, high, and middle) and various lipid classes.

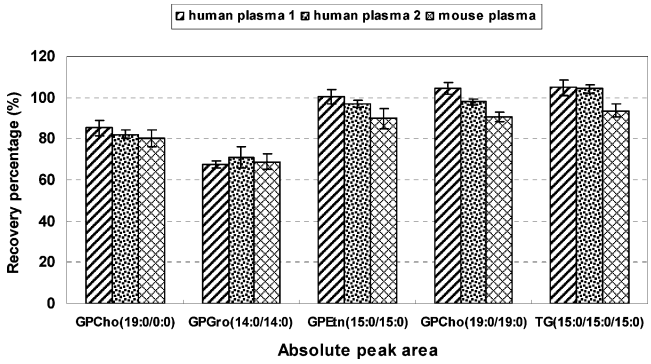

Figure 3. Recoveries of five nonendogenous lipids of the validation mixture. The recovery was calculated for each lipid standard as the ratio of the peak area of that lipid spiked before extraction to that spiked after extraction, and multiplied by 100. Three different plasma samples were extracted in triplicate and analyzed in duplicate. The plasma samples were spiked with the five nonendogenous lipids at an intermediate concentration level (see section 2.3) both before and after extraction. The error bars represent the RSD of the recoveries.

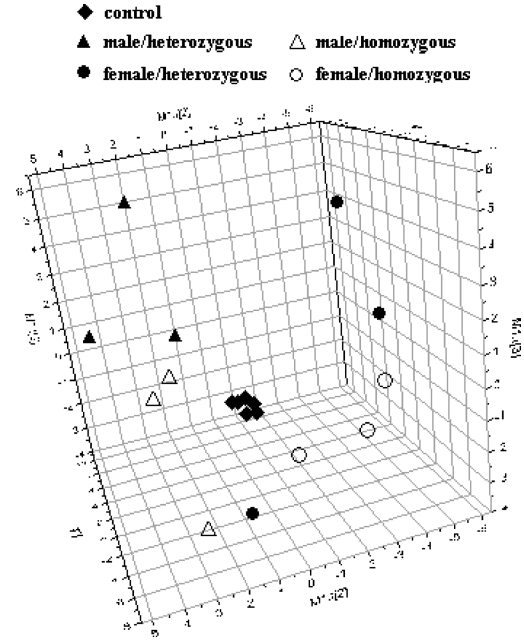

Figure 4. 3D Score plot of principal component analysis of mean centered data with gender/genotype information.

of Table S1 in the Supporting Information, but these ratios were not calculated in that table).

In conclusion, the reported RPLC-ESI-LTQ-FTMS method was fully validated, also the intermediate precision was determined, which was not reported by Barroso and Bischoff[26] and Laaksonen et al.[23] The performance of our method was good, and at least as good as the methods reported before.[23] For example, the linearity was satisfactory, which was for some unknown reasons suboptimal for the UPLC-TOF-MS method reported by Laaksonen et al.[23] for lipid standards of GPCho (17:0/17:0), GPCho (17:0/0:0), TG (17:0/17:0/17:0), GPCho (16:0/16:0, D6), TG (16:0/16:0/16:0, 13C3) at the concentration range of 10−250 μg/mL. In addition, the peak shapes and separation of PLs and TGs was in our method better than reported before.[8,23,26,29]

3.2. Lipid Identification. Lipids detected in the human and mouse plasma was provisionally identified using the high accuracy mass values of the molecular ion (positive ion mode, m/z range 400−1300) delivered by RPLC-ESI-LTQ-FTMS. MS/MS fragmentation, acquired with the linear ion-trap because of the faster acquisition times, was used to obtain structural information. The observed accurate masses were used for preliminary identification using "Lipidsearch"[39] with a mass tolerance of less than 10 ppm in order to cover all possible candidates based on the elemental composition at the very start of the identification process. By accurate mass and MS/MS data, in total 163 lipid species were identified representing eight lipid classes: lyso-GPChos, lyso-GPEtns, SMs, GPEtns, GPChos, DGs, ChoEs and TGs. Data obtained from the identification is available in Table S3 in the Supporting Information. For the comparison of samples (see below), the peak area of the most abundant 92 lipid species were determined using reconstructed extracted ion chromatograms acquired with LTQ instead of FTMS, as the present version of the quantitation software could not handle the high resolution data.

3.3. Method Application: Lipid Profiling of Mouse Plasma. After successful validation of the method, lipid profiling was applied to plasma samples obtained from 12 p53 mutant mice to demonstrate the potential of the developed method. P53 is

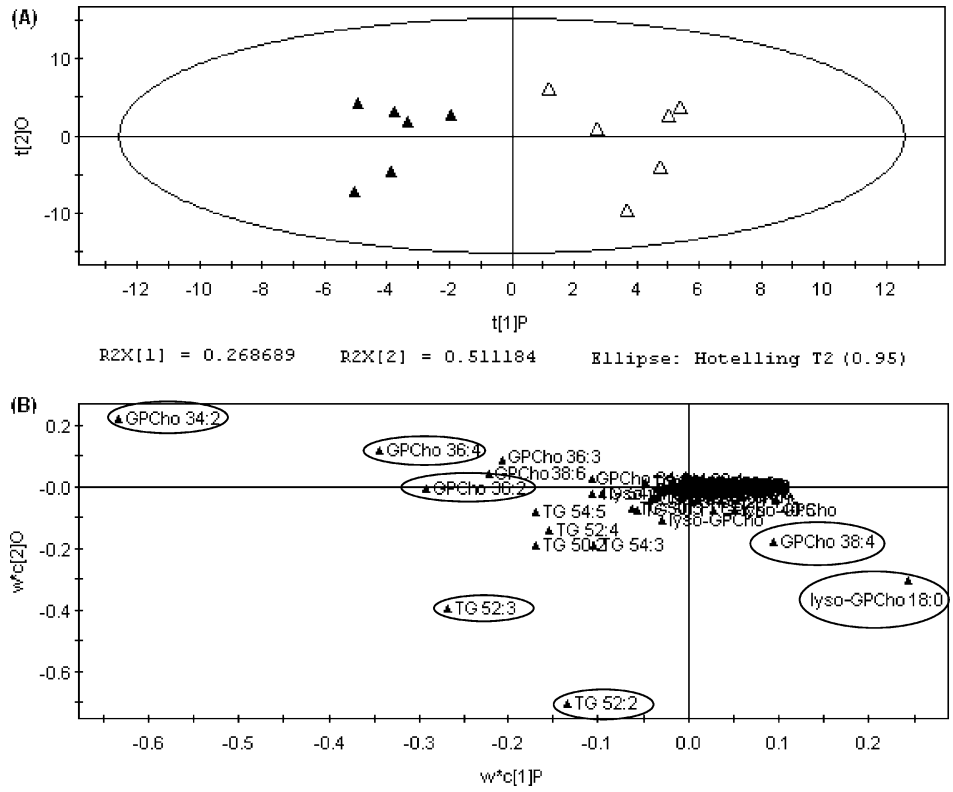

Figure 5. (A) Score and (B) loading plots from O-PLS DA to differentiate between gender using mean-centered data. Assignment: (A) ▲, male mice; Δ, female mice. (B) Lipid species marked with ovals contributed to the separation of male and female mice.

a transcription factor that regulates the cell cycle and apoptosis, and which functions as a tumor suppressor. Recent studies have suggested that p53 is also involved in the regulation of metabolism.[40,41] The mutant mice used for our study carried the modified p53 gene in either one allele of the chromosome or in both alleles of the chromosome (heterozygous or homozygous selectively).

Six male and six female p53 mutant mice were analyzed, six of them were heterozygous, and six homozygous mice. Including QC samples, in total 36 samples were analyzed. In the sequence, one blank sample (water extracted according the sample pretreatment protocol) was injected after every fifth sample. No problems were observed during the whole series; no significant carryover of lipids was observed, and no significant change in the pressure profile during the solvent gradient, typically from 160 to 205 bar at the end of the gradient, was observed. The carryover was evaluated by injecting a blank sample after injecting a plasma sample which was enriched 5 times more compared to the standard sample preparation procedure. The most intensive peaks were selected from the base peak chromatogram, and the peak areas from the smoothed extracted ion chromatograms in the blank and concentrated plasma sample were compared. The peak areas of lipid species like monoacyl lipids and phospholipids in the blank were far less than 1% of those found in the concentrated sample. Most peak area of TGs was also less than 1%; very few of them were found between 1% and 5% of the concentrated sample. The test was performed at several occasions, and the results were found to be consistent. The variation (expressed as standard deviation) in retention time of the five IS during the 36 samples analysis was 0.01−0.04 min (see Table S4 in Supporting Information). For the QC samples (n = 6, each extracted individually and injected in duplicate), the RSD of the signals of 11 randomly selected lipids (see section 3.1.2) corrected by the response of the corresponding IS were lower than 10% (see Figure S2 in the Supporting Information). In conclusion, the developed RPLC-ESI-LTQ-FTMS method appeared suitable for the analysis of a larger number of samples.

After normalization to the appropriate internal standard and averaging of duplicates, the selected 92 lipids present in all samples were subjected to principal component analysis (PCA)[42] (Figure 4) and orthogonal partial least-squares data analysis (O-PLS DA)[43] (Figures 5 and 6). In the 3D-PCA score plot of the mean centered data, the QC samples, that consisted of a pooled sample of all mouse samples, were located in the center of the plot close to each other (Figure 4). A clear separation of the samples due to different gender was observed. Heterozygous mice were also to some extent separated from homozygous mice, but some overlap between these two groups was observed.

In the mice model, or in complex biological systems in general, various variations are present (gender, transgenic, homozygous/heterozygous and probably also more), and at the same time, a large range of endogenous metabolites in the biofluid with varying concentrations are analyzed. Therefore, differences in variation, which are not too dominant, may not show up during unsupervised data analysis such as PCA.[21,44] Therefore, supervised O-PLS DA analysis (a rotated PLS model) was carried out to investigate whether differences between the different classes could be found between male and female mice as well as between heterozygous and homozygous mutant mice. O-PLS DA was able to discriminate according to gender as can be seen from the score plot (Figure 5A), and various lipids could be identified which contributed to the separation

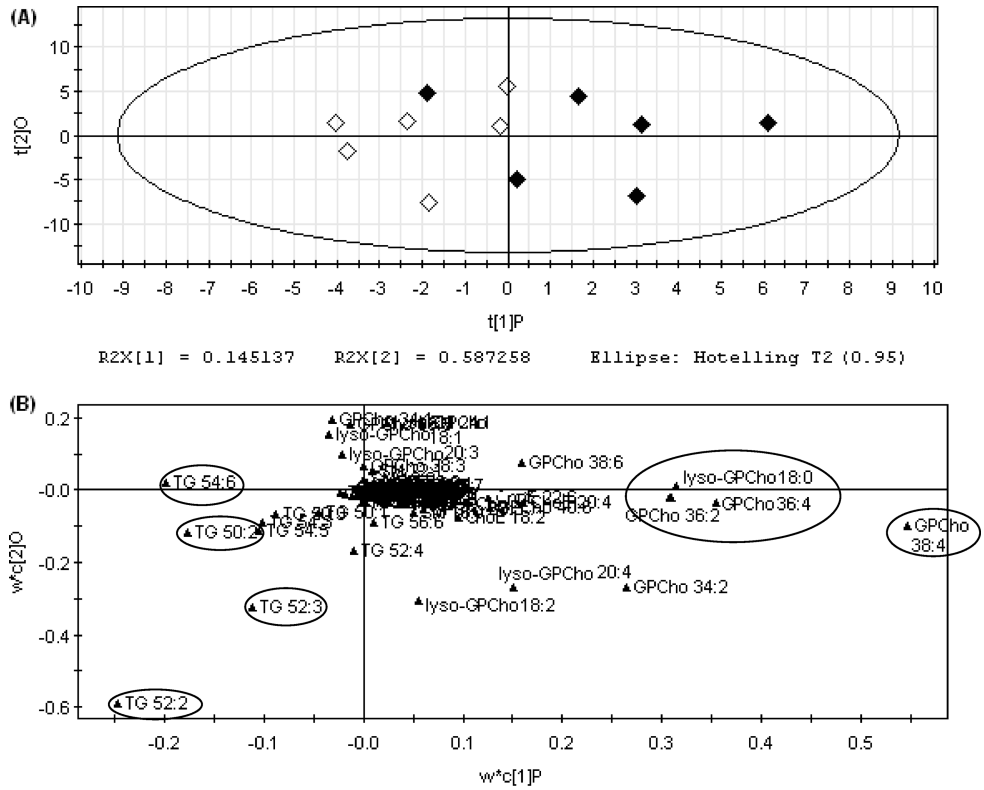

Figure 6. (A) Score and (B) loading plots from O-PLS DA to differentiate between genotype using mean-centered data. Assignment: (A) ◊, homozygous mice; ◆, heterozygous mice. (B) Lipid species marked with ovals contributed to the separation of homozygous and heterozygous mice.

according to gender (Figure 5B); PLs of GPCho (34:2), GPCho (36:4) and GPCho (36:2) were found more abundant in male mice, while GPCho (38:4) and GPCho (18:0/0:0) were found more abundant in female mice. Several TGs (indicated with black ovals in Figure 5B) were found more abundant in two male mice. An O-PLS DA model to differentiate between the genotype was able to separate the homozygous rather well from the heterozygous mice, except for one heterozygous mouse that was within the group of homozygous mice (Figure 6A). The O-PLS DA loadings plot (Figure 6B) highlighted the lipid species which contributed most to the separation of homozygous from heterozygous mice. TG (52:2), TG (54:6), TG (50:2) and TG (52:3) were found to be more abundant in homozygous mice and GPCho (38:4), GPCho (36:4), GPCho (36:2) as well as GPCho (18:0/0:0) were found more abundant in mice heterozygous for the p53 mutation. Statistical significance of the obtained gender and phenotype differences between the mice including the discriminating lipids was checked by using cross-validation and permutation tests. In conclusion, these preliminary results indicate a relation between p53 expression and lipid profiles; however, further experiments are required to allow a more solid biological interpretation.

4. Conclusions

A method for profiling of lipids in human and mouse plasma was successfully developed, validated and applied in a mutant mice study. The method allows the profiling of over 160 lipids belonging to eight different lipid classes in a single RPLC-ESI-LTQ-FTMS run. The chromatography is performed using a fused-core C_8 column (with 2.7 μm fused-core silica particles and a 0.5 μm thick porous shell) allowing higher flow rates and thus faster separations without loss of column efficiency. The chromatographic separation was comparable and to some extent better than those so far reported in the literature and FTMS detection proved to be robust in the analysis of a series of 36 biological samples. The ESI-LTQ-FTMS appeared to be essential in the identification process of the individual lipids and performed well for quantitative profiling. The analytical characteristics of the method for the analysis of plasma, that is, linearity, limit of detection, repeatability, intermediate precision, and recovery, were satisfactory. Furthermore, the validated method was successfully applied to the analysis of plasma samples from p53 mutant mice. The result showed that, based on lipid profiles, there was a clear distinction between male and female mice and there was some separation between heterozygous and homozygous mice. This validated method will be used in our systems biology approaches to measure changes in levels of endogenous metabolites in human and animal body fluids after exposure to drugs or complex mixtures such as Traditional Chinese Medicine.

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Supporting Information Available: Figure S1, extracted ion chromatogram of RPLC-ESI-LTQ-FTMS of several typical lipid species in mouse plasma; Figure S2, RSD of signal of 11 randomly selected lipid species obtained as ratio of the peak areas of the lipid and the corresponding IS in the QC samples. Tables of slope, intercept and R^2 of the calibration lines for the five lipids of the validation mixture, estimated injection concentration of randomly selected peaks for precision experiment, lipid identification based on MS/MS fragments and very high accurate masses, and variation of the retention time for five lipids used as internal standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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