Title

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Journal

Analytical Biochemistry, 455(1)

ISSN

0003-2697

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Publication Date

2014-06-01

DOI

10.1016/j.ab.2014.03.019

Analytical Biochemistry 455 (2014) 48–54

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Sample preparation and orthogonal chromatography for broad polarity range plasma metabolomics: Application to human subjects with neurodegenerative dementia

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ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 22 March 2014
Accepted 28 March 2014
Available online 4 April 2014

Keywords:
Metabolomics
Sample preparation
Orthogonal chromatography
Mass spectrometry
Dried plasma spot

ABSTRACT

We describe a simple protocol for the preparation and orthogonal hydrophobic/hydrophilic LC–MS/MS analysis of mouse and human plasma samples, which enables the untargeted ("shotgun") or targeted profiling of hydrophilic, amphipathic, and hydrophobic constituents of plasma metabolome. The protocol is rapid, efficient, and reliable, and offers several advantages compared to current procedures. When applied to a training set of human plasma samples, the protocol allowed for the rapid acquisition of full Log*P* metabolic profiles in plasma samples obtained from cognitively healthy human subjects and age-matched subjects with mild cognitive impairment or Alzheimer's disease (n = 15 each). Targeted analyses confirmed these findings, which are consistent with data previously published by other groups.

Despite its irreplaceable role in biomarker discovery, blood plasma presents multiple unresolved challenges for metabolomics-based analysis. Three key factors contribute to the analytical complexity of this biological matrix: (i) the occurrence of interfering proteins, (ii) the vast chemical diversity of its small-molecule constituents, and (iii) the wide range of concentrations at which such constituents are present. The removal of proteins from plasma has been achieved using a variety of approaches [1,2]. These include "two-phase" extraction, in which proteins are precipitated with a water-miscible organic solvent (e.g., methanol or acetonitrile) and then eliminated by centrifugation; solid phase extraction by low-pressure chromatography; and "three-phase" extraction [3], in which polar and apolar analytes are differentially separated using a combination of water-miscible and water-immiscible organic solvents (e.g., methanol and chloroform) [3]. Each of these approaches presents distinct advantages and limitations. For example, two-phase extraction methods are fast, inexpensive, and reliable, but often yield samples that contain substantial amounts of phospholipids, peptides, and small proteins. Solid-phase extraction, on the other hand, generates samples that are enriched in specific classes of analytes, but is inappropriate when the objective is metabolomic profiling. A compromise solution is represented by three-phase extraction methods, which are used for the extraction of plasma metabolites with a broad range of polarities. In addition to these well-known approaches, the use of paper as a solid support for the extraction of small molecules from blood or plasma has recently attracted a great deal of interest [4–6]. This strategy is not immediately suitable for high throughput implementation, but has already reached routine laboratory analysis thanks to its efficiency and reliability. An additional factor that contributes to the analytical complexity of human plasma is the remarkable qualitative and quantitative diversity of the chemical entities found in this biological matrix. The current version of the Human Metabolome Database (3.5) reports that human blood contains 4528 different compounds [7,8]. From an analytical perspective, this diversity implies that a broad spectrum of analyte polarities (with Log*P* values ranging from <−2 for sugars and basic aminoacids to >10 for triacylglycerols) and concentrations (from millimolar to femtomolar) must be taken into consideration. This represents, in turn, a considerable obstacle to LC–MS-based analyte identification

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http://dx.doi.org/10.1016/j.ab.2014.03.019
0003-2697/

and quantification, mainly due to ion suppression effects and limited linearity range of MS detectors. A case in point is provided by the phospholipid (PC),[1] which is highly abundant in human plasma and displays a high ionization efficiency in positive ESI-MS: the ability of the phosphoryl-choline headgroup of PC to generate strong MS/MS ion currents (at $m/z = 184.07$) interferes with the detection and quantification of many coeluting plasma components. The present study describes a protocol that enables the rapid shotgun profiling of plasma analytes of broadly different polarities. Plasma samples collected from mice or human subjects are separated into three fractions using a slight variation of the three-phase Bligh–Dryer extraction [3]. The fractions are then subjected to an orthogonal two-column/three-gradient chromatographic procedure on reversed-phase or HILIC columns [9,10]. The protocol permits the exploration of a polarity space that ranges from sugars (Log$P < -2$) to triacylglycerols (Log$P > 10$). The results of a nontargeted application of the present protocol to human subjects with neurodegenerative dementia, which were confirmed by subsequent targeted analyses, are in good agreement with literature data documenting the existence of sphingolipid alterations in plasma of cognitively impaired people.

Materials and methods

Human subjects

We included 45 male and 45 female subjects [30 with Alzheimer's Disease (AD), 30 with mild cognitive impairment (MCI), and 30 cognitively healthy controls] from the MCI/AD Italian prevention project [11,12] aimed at studying cognitive and neuropsychiatric symptoms and disorders in patients with MCI and AD, at the IRCCS Santa Lucia Foundation memory clinic in Rome, Italy. Table 1 shows the socio-demographic and clinical characteristics of the three groups of AD, MCI, and healthy controls separately for males and females. There were no differences among the three groups in age and gender. All subjects underwent examination between January 2008 and January 2012, including a thorough clinical neurological and psychiatric examination and extensive cognitive assessment, and were included at first diagnosis of the disease, and before treatment with psychotropic drugs or acetylcholinesterase inhibitors; patients who received such medications were excluded from the study. A thorough clinical examination was used to exclude patients with secondary cognitive deficits due to somatic disorders such as unbalanced diabetes, coronary heart disease, or other major medical illnesses causing secondary cognitive impairment. Patients with potential vascular impairment were excluded if they had Hachinski Ischemic scale score > 4 or MRI evidence of white matter lesions identified through consensus by a neuropsychologist expert in neuroimaging and a neuroradiologist. Cognitive impairment on the tests was defined using normative data for the Italian population [13], with specific age and educational norms. Diagnosis of AD was made by trained clinical psychiatrists or neurologists, using criteria consistent with those outlined by NINCDS–ADRDA [14]. In the current analysis we included only patients with mini-mental state examination (MMSE) score $\geqslant 18$ and clinical dementia rating (CDR) = 1 to ensure that only mild AD patients were included. Diagnosis of MCI was made according to established criteria [11,15] by trained clinical neurologists and psychologists. The 30 volunteers included in the control group were recruited from the general population of the same geographic area (Italian administrative region of Lazio) as the patients. All controls were living independently in their own homes, and performed at a normal level of cognitive functioning in all neuropsychological tests according to age and education norms for the Italian population [13]. The nature and purpose of the study were presented to patients and caregivers, and controls, and written informed consent was obtained. The study was approved by the Ethical Committee of the Santa Lucia Foundation.

Reagents, standards, and instruments

Solvents and chemicals were purchased from Sigma Aldrich (Milan, Italy). Mouse plasma was from Tebu-Bio (Le Perray-en-Yvelines, France) and sphingomyelin standards were from Avanti Polar Lipids (Alabaster, AL, USA). UPLC/MS and MS/MS systems and columns were from Waters (Milford, USA).

Human plasma samples

Blood samples were taken by venipuncture in the morning after an overnight fast. Blood was collected into 10 ml tubes containing spray-coated K2EDTA (Vacutainer, Becton Dickinson) and plasma was obtained by centrifugation at 400*g* for 15 min, divided into 1 ml sterile cryovials, and then stored at −80 °C. Blood drawing and sample preparation of human plasma samples were carried out applying the best safety precautions and gloves and goggles were used for sample handling.

Sample preparation

Human or mouse plasma samples (0.1 ml) were transferred to glass vials. Liquid–liquid extraction (LLE) was carried out using a mixture of methanol/chloroform/heptane (2/0.5/0.5 by volume; 0.3 ml). Human plasma samples for shotgun metabolomics analysis were extracted with the same mixture described above but spiked with unnatural ceramide (d18:1/17:0) at 2 μM concentration as internal standard for data normalization. After mixing for 30 s with a Vortex, chloroform (0.1 ml) and water (0.1 ml) were sequentially added, thoroughly stirring after each addition. The samples were centrifuged for 15 min at 3500*g* at room temperature and the acqueous (upper) and organic (lower) phases, separated by a protein disk, were collected separately. The upper phase was split into two equal portions, referred to hereafter as Fraction C and Fraction B. The lower phase is referred to as Fraction A. The samples were dried under a stream of N_2 and the residues were dissolved using (a) water containing ammonium formate (10 mM, adjusted to pH 5 with formic acid; 50 μl) for the analysis of Fraction C; (b) pure methanol (0.1 ml) for the analysis of Fraction B; and (c) methanol/isopropyl alcohol/chloroform (1/1/1, by volume; 0.2 ml) for the analysis of Fraction A. After mixing for 30 s, the samples were transferred to glass vials, centrifuged for 10 min at 3500*g* at room temperature, and transferred to glass vials for LC–MS/MS analyses. Before injection, Fraction C was diluted with 50 μl of 10 mM ammonium formate in acetonitrile/water (95/5, by volume). Blank procedure samples were prepared exactly as the samples, but replacing plasma with phosphate-buffered saline solution (50 mM). These samples, acquired before and after each human sample group, were used as quality controls. Retention time and peak areas of the internal standard were used to check instrument response variability and retention time shifts. The amount of plasma used (0.1 ml) enabled several replicate injections in both ESI+ and ESI− ion modes. In some experiments (solid–liquid-extraction procedure) mouse plasma samples (0.1 ml, split into 4×25 μl aliquots) were deposited onto Bond Elut DMS paper (Agilent Technologies, Palo Alto, CA, USA) and allowed to dry for 1 h at room temperature. The 25 μl spots were individually cut, transferred to a glass vial, and processed as described above, with the following modifications. A methanol/

[1] *Abbreviations used:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol; CE, cholesteryl ester; PI, phosphatidylinositol; Cer, ceramide; HILC, hydrophilic interaction liquid chromatography; UPLC, ultra performance liquid chromatography; XIC, extracted ion current.

Table 1
Sociodemographic and clinical characteristics of males and females with Alzheimer's disease, mild cognitive impairment, and healthy control subjects.

	HC		MCI		AD	
Gender	Males	Females	Males	Females	Males	Females
Age	73.3 ± 3.5	73.3 ± 2.3	72.3 ± 3.7	73.1 ± 2.9	73.7 ± 3.0	74.5 ± 3.7
Education	13.4 ± 5.6	10.7 ± 4.0	10.3 ± 4.2	6.7 ± 2.8	9.6 ± 5.3	7.1 ± 4.6
MMSE	28.5 ± 2.0	29.1 ± 1.2	26.5 ± 1.7	26.3 ± 1.8	21.3 ± 4.6	20.0 ± 4.6

chloroform/heptane mixture (2/0.5/0.5 by volume, 0.3 ml) was added to the spots and stirred for 30 s on a Vortex. Chloroform (0.1 ml) and water (0.2 ml) were added, stirring after each addition. The samples were centrifuged for 15 min at 3500*g* at room temperature and the acqueous and organic phases were dried and resuspended as described above. For comparative experiments, mouse plasma samples (0.1 ml) were subjected to standard protein precipitation procedures ("crash") with organic solvents. The samples were diluted with acetonitrile or methanol (0.5 ml), mixed for 30 s with a Vortex, and centrifuged for 15 min at 3500*g* at room temperature. The supernatants were divided into three aliquots, dried under N_2, and resuspended as described above.

Liquid chromatography

Analyses were conducted on a UPLC Acquity system coupled to a Synapt G2 QToF mass spectrometer. Fraction *C* samples were loaded onto a 2.1 × 100 mm HILIC column eluted at 0.4 ml/min. The following gradient conditions were utilized: *A* = 10 mM ammonium formate in water, *B* = 10 mM ammonium formate in 95/5 acetonitrile/water. After 1 min at 100% *B*, a linear gradient was applied to 60% *B* in 11 min, followed by 1 min at 60% *B* and reconditioning to 100% *B*. Total run time was 16 min. Fractions *B* and *A* were analyzed using a C18 T3 column run (2.1 × 100 mm) at a flow rate of 0.4 ml/min with acetonitrile and isopropyl alcohol gradient systems, respectively. The following gradient conditions were utilized. Fraction *B*: *A* = 10 mM ammonium formate in water, *B* = 10 mM ammonium formate in 95/5 acetonitrile/water; after 1 min at 5% *B*, a linear gradient was applied to 100% *B* in 12 min, followed by 1 min at 100% *B* and reconditioning to 5% *B*; total run time was 18 min. Fraction *A*: *A* = 10 mM ammonium formate in 60/40 acetonitrile/water, *B* = 10 mM ammonium formate in 90/10 isopropyl alcohol/acetonitrile; after 1 min at 30%, solvent *B* was brought to 35% in 3 min, then to 50% in 1 min, and then to 100% in 13 min, followed by a 1 min 100% *B* isocratic step and reconditioning to 30% *B*. Total run time was 22 min [16,17]. Injection volume was set at 2 µl for Fraction *A* and 5 µl for Fractions *C* and *B*.

Mass spectrometry

The capillary voltages were set at 3 and 2 kV for ESI+ and ESI−, respectively. The cone voltages were set at 30 V for ESI+ and 35 V for ESI−, respectively. The source temperature was 120 °C. Desolvation gas and cone gas (N_2) flow were 800 and 20 L/h, respectively. Desolvation temperature was 400 °C. Data were acquired in MS^e mode [18,19] with MS/MS fragmentation performed in the transfer region. Low energy scans were acquired at fixed 4 eV potential and high energy scans were acquired with an energy ramp from 25 to 45 eV. Scan rate was set to 0.3 s per spectrum. Scan range was set to 50 to 1200 *m*/*z*. Leucine enkephalin (2 ng/ml) was infused as lock mass for spectra recalibration.

Analyte identification

Analytes were identified by interrogating the METLIN [20,21], HMDB [7,8] and LipidMaps [22,23] databases. Tolerance on *m*/*z* values was set to 3 ppm. Identification was based on both accurate mass and calculated brute formula matching and confirmed with tandem mass data. Log*P* data from the HMDB file of each identified metabolite were used.

Multivariate data analysis

Raw data from high resolution shotgun metabolomics LC/MS runs were subjected to principal component analysis (PCA) [24] using the MarkerLynx software (Waters Inc.). Accurate mass and retention time data of observed metabolites, previously normalized and realigned using the internal standards peak areas and retention times, were included in the multivariate analysis and assigned as *X*-variables. The following parameters were used to derive the Scores plots reported in Fig. 4: scan range 100 to 1200 *m*/*z*; extracted ion current windows 30 mDa; no smoothing; Pareto scaling applied; no transformations; maximum 5 components considered. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) [25,26] was used to identify metabolites differently expressed between different experimental groups. GraphPad Prism software (GraphPad Software, Inc., USA) was used for statistical analyses of targeted data.

Targeted sphingomyelin quantification

Plasma samples (20 µl) were diluted to 0.1 ml with distilled water and subjected to sample preparation as described above. Reconstituted hydrophobic fractions (Fractions *A*) (5 µl) were analyzed using a UPLC-Xevo Triple-Quadrupole MS system. Sphingomyelin species SM(d18:1/16:0), SM(d18:1/18:0), SM(d18:1/24:0),

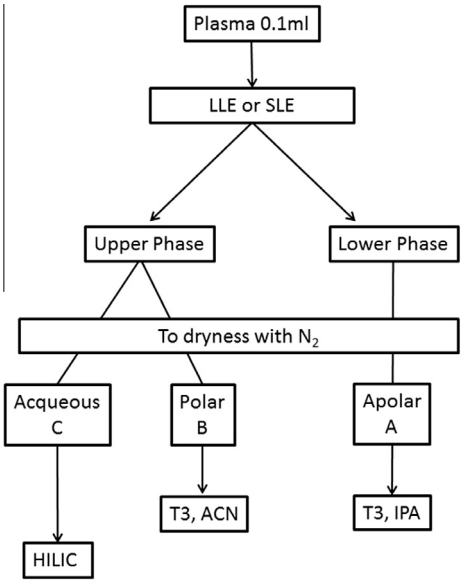

Fig.1. Schematic representation of the sample preparation protocol described in the present study. Each step was designed to emphasize separation between hydrophobic and hydrophilic analytes. Abbreviations: ACN, acetonitrile; IPA, isopropanol; LLE, liquid–liquid extraction; SLE, solid–liquid extraction.

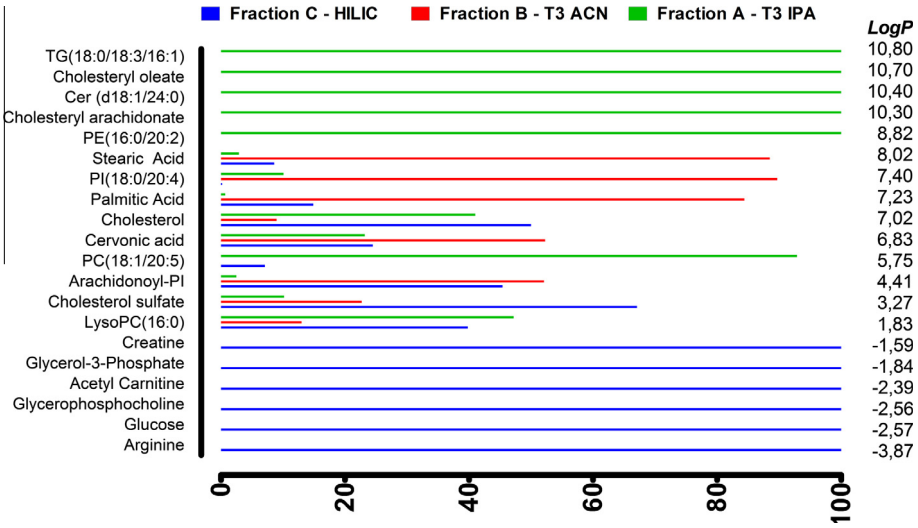

Fig.2. Distribution of 20 representative analytes in Fraction *A* (green), Fraction B (red), and Fraction C (blue). Calculated Log*P* values are reported on the right of each analyte. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

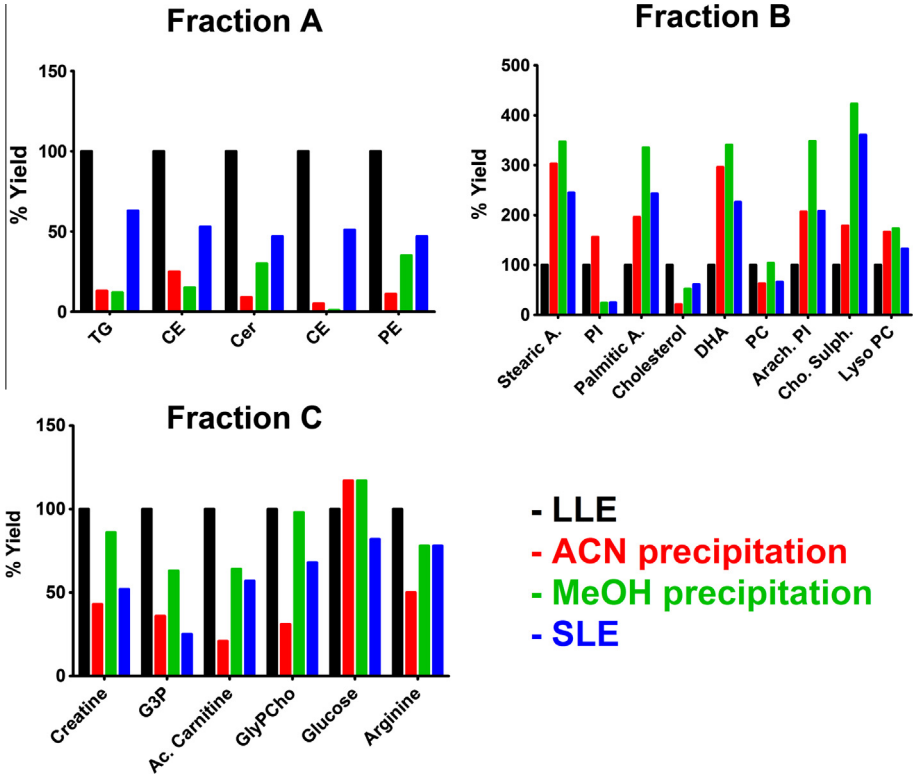

Fig. 3. Extraction efficiency of the new method, as liquid–liquid extraction (LLE, black) or solid–liquid extraction (SLE, blue) compared to the acetonitrile (red) and methanol (green) precipitation procedures. The percentage yield was measured using extracted ion currents, with the LLE procedure as reference set at 100%. Experiments were run in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and SM(d18:1/24:1) were separated using a linear gradient from 70% *A* to 100% *B* in 6.6 min followed by 1 min at 100% *B* and reconditioning to 70% *A*; total run time was 9 min on an ACQUITY UPLC BEH C18 column (2.1 × 50 mm) at 0.4 ml/min flow rate (*A* was water/acetonitrile 80/20 plus 0.1% formic acid, *B* was acetonitrile/isopropyl alcohol 20/80 plus 0.1% formic acid). Sphingomyelin species were quantified on the basis of their MRM traces, using a calibration curve ranging from 1 nM to 5 μM. The unnatural lipid (23:0;23:0) PC was included in the extraction process as internal standard (0.5 μM).

Results and discussion

Fig. 1 summarizes the analytical procedure described in the present report. A slight variation of the classical three-phase Bligh–Dryer extraction [3] was used to divide plasma samples into three fractions (termed *A*, *B*, and *C*), which were analyzed separately using either reversed-phase chromatography (Fractions *A* and *B*) or HILIC chromatography (Fraction *C*) coupled to high-resolution MS/MS. Fig. 2 illustrates the distribution of 20 representative analytes, selected on the basis of their biogenetic diversity and chemical

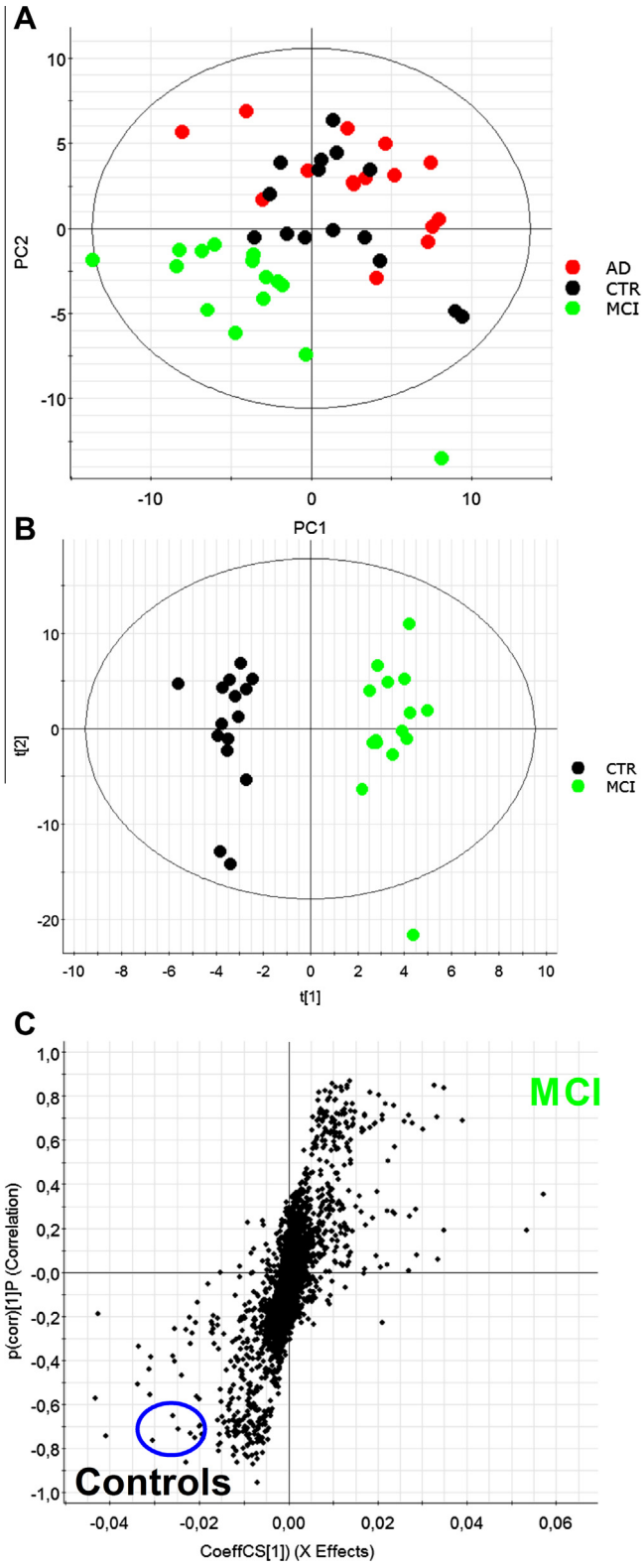

Fig.4. Principal component analysis of plasma samples (Fraction *A*, ESI+ mode) from healthy female subjects, female subjects with MCI, and female subjects with AD ($n = 15$, matched years: 77 ± 2). (A) Scores plot of samples from AD, MCI, and age-matched cognitively healthy controls. A moderate separation was observed between MCI and controls. (B) Scores plot from OPLS-DA analysis of healthy controls versus MCI patients. (D) Scatter plot of variables (m/z, RT values) showing increasing contribution to controls (toward bottom left) and MCI patients (toward top right). Dots corresponding to sphingomyelin species are symbolized with a blue circle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

heterogeneity, in each of the three fractions. Table 2 provides a list of those analytes along with their MS properties, calculated Log*P*, and database reference identification. As shown in Fig. 2, Fraction *C* was exclusively populated by small hydrophilic molecules, such as amino acids and phosphorylated glycerols, which eluted from the HILIC column at water concentrations up to 40%. Fraction *B* contained midpolarity analytes (from Log*P* 1.5 to Log*P* approximately 8), including long-chain fatty acids and hydroxylated fatty acids, which were effectively separated by reversed-phase chromatography (using an acetonitrile gradient) and were detected by MS in the ESI^- mode. Fraction *A* included lipids of various polarities, from amphipathic species such as PC to highly apolar species such as triacylglycerols. We compared the present protocol to methanol and acetonitrile precipitation procedures widely utilized in metabolomics experiments [27,28]. Extraction yields for each procedure were estimated based on the sum of XIC peak areas of representative analytes derived from their chromatographic separations over the three LC systems. Fig. 3 shows the results of these comparative tests. For Log*P* values above 8, our protocol was ostensibly superior to both acetonitrile and methanol precipitation methods: as expected, highly apolar lipid species (e.g., TG, PE, CE, and ceramides) were not efficiently extracted in the absence of a suitable organic solvent. Our procedure was also superior to two-phase protein precipitation in the ability to extract highly hydrophilic compounds (Log*P* < 0). On the other hand, methanol precipitation was more efficient in extracting amphipathic molecules in the midpolarity range, such as fatty acids, PI, and cholesteryl sulfates (Fig. 3). Interestingly, however, this superiority did not hold for a solid-supported variant of our method, which provided extraction yields for these analytes that were comparable to those obtained using methanol crash. Notably, the addition of trifluoroacetic acid (1%, 5 µl per sample) prior to the liquid extraction markedly lowered the amount of lyso-PC present in the acqueous phase after extraction, thus reducing the risk of ion suppression during LC/MS analysis. For example, with this simple expedient the spreading of stearoyl-LysoPC (524.3719 m/z) in LC runs of Fraction *A* was reduced from 18 to 1%. As a first test of the newly described procedure, we analyzed a training set of human plasma samples obtained from cognitively healthy elderly female subjects along with an equal number of samples from age-matched female subjects suffering from mild cognitive impairment (MCI) or Alzheimer's disease (AD) ($n = 15$ for each group). Shotgun data for Fractions *A*, *B*, and *C* generated from each subject were acquired in both positive and negative ion modes. Multivariate data analysis tools were used to explore the existence of potential differences between the three groups, using several hundreds of accurate mass/retention time data pairs as variables for PCA. Though the full Log*P* range of each sample was investigated, only representative data are discussed here. Additional findings will be discussed in subsequent publications. Fig. 4A shows the scores plot of Fraction *A* (apolar phase) acquired in the positive-ion mode. Samples, clustered by experimental group, are reported in the plot as colored dots (observations). A clear separation between female subjects with MCI (green dots) and control subjects (black dots) was noted. Conversely, no obvious separation was observed between subjects with AD and controls. Control and MCI groups were further compared using OPLS-DA (Fig. 4B) searching for analytes differentially represented in the two groups. From the corresponding scatter plot (Fig. 4C), among various signals that are currently under investigation, a group of variables, corresponding to several sphingomyelin species ranging from (d18:1/18:0) to (d18:1/24:1) (blue circle) appeared to be higher in the control group with respect to MCI. To further probe these results, which are suggestive of an alteration in plasma sphingolipid levels in elderly women with MCI, we subjected Fraction *A* to a targeted analysis using an LC–MS/MS method

Table 2
Representatives analytes present in mouse plasma selected for use as markers in method evaluation.

Dbase ref.	*m/z*	Adduct	Analyte	Log*P*
HMDB00517	175.119	[M+H]+	Arginine	−3.87
HMDB00122	198.0972	[M+NH_4]+	Glucose	−2.57
HMDB00086	258.1106	[M+H]+	Glycerophosphocholine	−2.56
HMDB00201	204.1233	[M+H]+	Acetylcarnitine	−2.39
HMDB00126	171.0057	[M−H]−	Glycerol-3-phosphate	−1.84
HMDB00064	132.0771	[M+H]+	Creatine	−1.59
HMDB10382	496.3401	[M+H]+	LysoPC(16:0)	1.83
HMDB00653	465.3043	[M−H]−	Cholesteryl sulfate	3.27
LMGP06050006	603.2938	[M−H_2O+H]+	1-Arachidonoyl-*sn*-PI	4.41
HMDB08083	806.5703	[M+H]+	PC(18:1/20:5)	5.75
HMDB02183	327.2329	[M−H]−	Cervonic acid	6.83
HMDB00067	369.3514	[M−H_2O+H]+	Cholesterol	7.02
HMDB00220	255.2326	[M−H]−	Palmitic acid	7.23
HMDB09816	885.5499	[M−H]−	1,Stearoyl-2,arachidonoyl-*sn*-PI	7.4
HMDB00827	283.2636	[M−H]−	Stearic acid	8.02
HMDB08934	744.5562	[M+H]+	PE(16:0/20:2)	8.82
HMDB06726	690.621	[M+NH_4]+	Cholesteryl arachidonate	10.27
HMDB04956	632.6365	[M−H_2O+H]+	Ceramide (d18:1/24:0)	10.35
HMDB00918	668.6367	[M+NH_4]+	Cholesteryl oleate	10.68
HMDB45105	872.7720	[M+NH_4]+	TG(18:0/18:3/16:1)	10.77

The table reports accurate *m/z* values and MS adduct for each analyte, along with its Log*P* value and database reference ID.

devised to quantify plasma sphingomyelin species. The results, reported in Fig. 5, confirmed that the levels of sphingomyelin (d18:1/16:0), (d18:1/24:0), and (d18:1/24:1) were significantly lower in plasma of female subjects with MCI compared to age-matched control subjects. Based on the results of these analyses, we hypothesized that similar changes might be detectable in plasma of male subjects. Therefore, we extended our targeted studies to cognitively healthy elderly male subjects as well as male subjects with MCI and AD (all 77 ± 2 years old, $n = 15$ per group). We found no statistically detectable differences between control subjects and subjects with MCI (Fig. 5). The analyses did reveal, however, a difference in the levels of sphingomyelins (d18:1/16:0), (d18:1/18:0), and (d18:1/24:1) between control subjects and subjects with AD (Fig. 5). To further probe these results, which are suggestive of an alteration in plasma sphingolipid levels in elderly women with MCI, we subjected Fraction *A* to a targeted analysis using an LC–MS/MS method devised to quantify plasma sphingomyelin species in both males and females. We used four separate two-way analyses of variance (ANOVAs) to highlight overall differences in d18:1/16:0, d18:1/18:0, d18:1/24:0, and d18:1/24:1 values (considered as dependent variables), using diagnostic groups (control subjects, MCI, and AD) and gender (males and females) as independent variables. The statistical threshold used in all ANOVAs was $P < 0.05$. Then, pairwise Fisher's PLSD post hoc tests were performed to assess differences in sphingomyelin species between individual diagnostic groups. There was a significant overall effect of the diagnostic group only for d18:1/16:0 ($F = 3.30$; $df = 2,75$; $P = 0.042$), and d18:1/24:1 ($F = 5.31$; $df = 2,75$; $P = 0.007$) and a significant overall effect of gender for all sphingolipids ($F > 17.0$, $df = 1,75$, $P < 0.0001$ for all effects). There was also a significant interaction between diagnostic groups and gender for all sphingolipids ($F > 12.0$, $df = 2,75$, $P < 0.0001$ for all effects). In particular, all sphingolipid levels were reduced in females in comparison with males. Further, d18:1/16:0 levels were reduced in MCI females ($P < 0.026$) and AD males ($P < 0.014$) in comparison with control subjects. Last, d18:1/24:1 levels were reduced in MCI females ($P < 0.002$) and AD males ($P < 0.030$) in comparison with control subjects. We did not perform pairwise post hoc analyses of d18:1/18:1 and d18:1/20:1 values because there was not an ANOVA overall effect of the diagnostic group for these sphingomyelin species. These results are in good agreement with those previously published by Han et al. [29], who reported that sphingomyelin species d18:1/22:1 and d18:1/24:1 are lower in plasma of subjects with AD (male plus female) relative to age-matched control subjects ($n = 26$ per group). It is important to point out that Han et al. did not separate their analytical sample by gender: thus, the gender difference potentially revealed by our analyses provides an element of novelty that deserves further investigation.

Conclusions

We describe a polarity-driven sample preparation protocol coupled to orthogonal hydrophobic/hydrophilic liquid chromatogra-

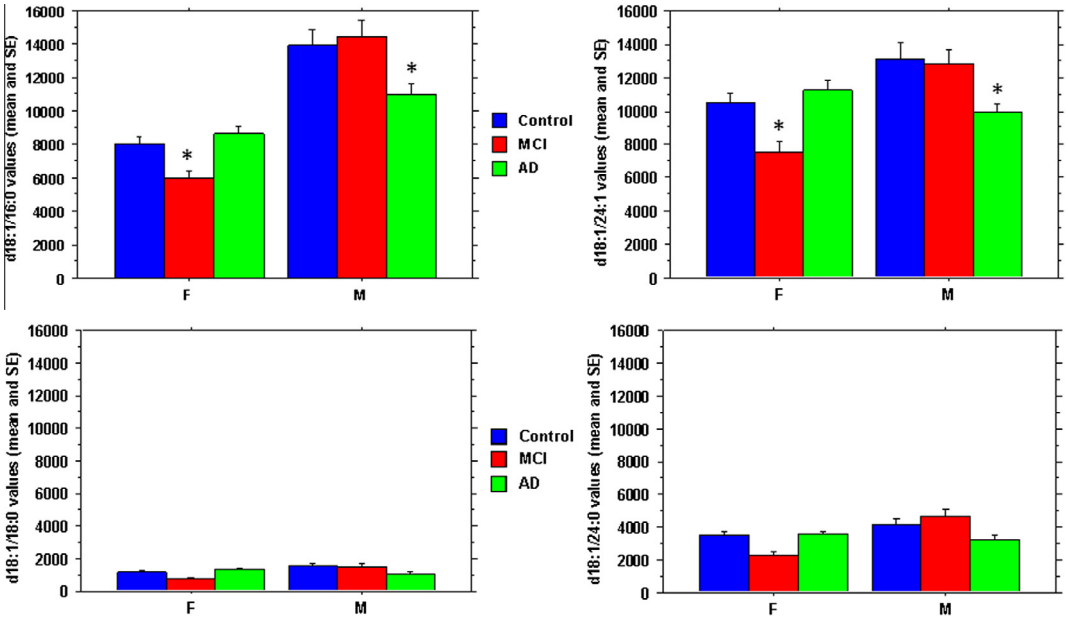

Fig.5. Results of targeted LC–MS analyses of various sphingomyelin (SM) species in plasma samples from male and female patients with MCI or AD and age-matched healthy control subjects. Data were analyzed using two-way (diagnosis by gender) ANOVAs followed by pairwise Fisher's PLSD post hoc tests (*$P < 0.05$ vs controls).

phy, which allows for the untargeted and targeted metabolomic investigation of human and mouse plasma. The analytes we selected to test the new procedure (Table 2) represent a broad range of Log*P* classes and metabolic pathways. Their wide Log*P* span (−3.87 to 10.77) demonstrates that virtually the entire chemical space of plasma is accessible to LC–MS/MS in just a few analytical steps and at a reasonable sample consumption (0.1 ml). For certain hydrophobic and hydrophilic analytes, the new protocol performs better than two widely used protein precipitation methods (with acetonitrile or methanol). Notably, the paper-supported version of the new protocol provides a valid analytical approach in the midpolarity range. In case a higher coverage of such metabolites is required, this option could be exploited with an acceptable increase of sample preparation time. Our first tests in human plasma samples suggest that the procedure can be successfully applied to the discovery of metabolomic alterations that accompany disorders of human cognition, such as MCI and AD.

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