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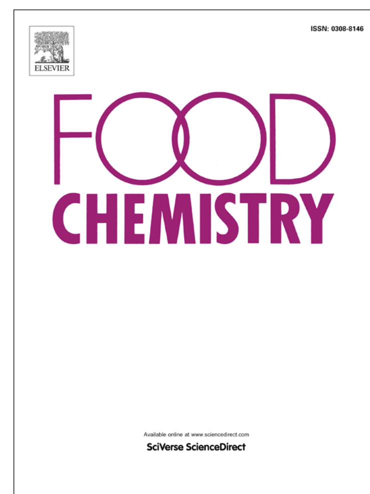

Phospholipidome of extra virgin olive oil: development of a solid phase extraction protocol followed by liquid chromatography - high resolution mass spectrometry for its software-assisted identification

Michela Antonelli, Barbara Benedetti, Chiara Cavaliere, Andrea Cerrato, Carmela Maria Montone, Susy Piovesana, Aldo Lagana, Anna Laura Capriotti

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1 **Phospholipidome of extra virgin olive oil: development of a solid phase**
2 **extraction protocol followed by liquid chromatography - high resolution mass**
3 **spectrometry for its software-assisted identification**

4

5 Michela ANTONELLI[1], Barbara BENEDETTI[1], Chiara CAVALIERE[1*], Andrea CERRATO[1],
6 Carmela Maria MONTONE[1], Susy PIOVESANA[1], Aldo LAGANA'[1,2], Anna Laura CAPRIOTTI[1]

7

8 [1] Department of Chemistry, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185
9 Rome, Italy

10 michela.antonelli@uniroma1.it, barabara.benedetti@uniroma1.it, chiara.cavaliere@uniroma1.it,
11 andrea.cerrato@uniroma1.it, carmelamaria.montone@uniroma1.it, susy.piovesana@uniroma1.it,
12 aldo.lagana@uniroma1.it, annalaura.capriotti@uniroma1.it

13 [2] CNR NANOTEC, Campus Ecotekne, Università del Salento, Via Monteroni, 73100 Lecce, Italy

14

15 * Corresponding author, email address chiara.cavaliere@uniroma1.it; phone + 39 06 4991 3834

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16 **Keywords**

17 Phospholipids, olive oil; Ultra-high performance liquid chromatography; High resolution mass

18 spectrometry; Lipidomics; Graphitized Carbon Black

19

20 **Abstract**

21 The determination of phospholipids in olive oil is challenging due to their low concentration. For

22 this reason, a comparison of two solid phase extraction procedures, namely weak anionic exchange

23 (WAX) and graphitized carbon black (GCB), is presented for the enrichment of phospholipids.

24 Analyses were performed by liquid chromatography-high resolution mass spectrometry (LC-

25 HRMS) and lipids were identified by Lipostar software. Compared to the WAX solid phase

26 extraction, GCB demonstrated the best performance and provided 82 identified phospholipids vs

27 only 32. The final method was validated for some representative phospholipids, showing good

28 repeatability and recovery (63-101%). High sensitivity was reached, with detection limits in the

29 range 9-36 ng g^{-1}, never reported before for phospholipids in olive oil. A semi-quantitative analysis

30 indicated phosphatidic acids and phosphatidylglycerols as the most abundant species, both in

31 number and concentrations. The GCB-LC-HRMS-Lipostar platform can be successfully applied for

32 a comprehensive polar lipidomic characterization of olive oils.

33

34 1. Introduction

35 Phospholipids are a wide class of amphiphilic substances characterized by a polar head and one or

36 two fatty acid chains. According to the hydrophilic part of the molecule, phospholipids are, in turn,

37 divided into phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS),

38 phosphatidylinositols (PI), phosphatidylglycerols (PG), lysophosphatidylcholines (LPC),

39 lysophosphatidylinositols (LPI) and phosphatidic acids (PA) (Fahy et al., 2005). Phospholipids are

40 one of the major constituents of cell membranes, therefore they are ubiquitously found both in the

41 animal and plant kingdom. In the recent years, several healthy properties have been ascribed to

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42 dietary phospholipids, such as antithrombotic, antiatherosclerotic and anti-inflammatory functions,
43 as well as role in reducing the risk of cardiovascular disease and blood cholesterol levels
44 (Karantonis, Antonopoulou, & Demopoulos, 2002; Küllenberg, Taylor, Schneider, & Massing,
45 2012). Furthermore, the composition of the fatty acid chains in the phospholipids structure plays a
46 key role in several biological function as antioxidant activity, memory increase and immunological
47 properties, as described in recent studies (Lordan & Zabetakis, 2017; Sun, Chen, Wang, & Lin,
48 2018). For these reasons, a raising interest has emerged for investigating phospholipids in food
49 matrices.
50 Extra virgin olive oil is a valuable food from the Mediterranean area, with specific organoleptic
51 characteristics as well as beneficial nutritional properties (Serra-Majem, Ngo de la Cruz, Ribas, &
52 Tur, 2003). It is mainly composed of triacylglycerols (ca. 99%), with polar lipids representing
53 minor components (<2%)(Alves, M. Domingues, & Domingues, 2018). It has been demonstrated
54 that the phospholipid content of extra virgin olive oil may provide precious qualitative and
55 quantitative data for evaluating its genuineness. ~~In fact, the concentration of these substances is
56 much lower compared with other vegetal oils, and a peculiar phospholipid composition can be
57 attributed to olive oil, thus providing a possible "fingerprint" for traceability and authenticity
58 studies.~~ In fact, the lower phospholipid concentration in the olive oil, compared to other vegetal
59 oils, allows to provide a distinct "fingerprint" for traceability and authenticity studies (Alves et al.,
60 2018; Gallina Toschi, Bendini, Lozano-Sánchez, Segura-Carretero, & Conte, 2013). For example,
61 the quali-quantitative analysis of phospholipids has been proven to be useful to identify the
62 adulteration of olive oil with hazelnut oil (Calvano, Ceglie, D'Accolti, & Zambonin, 2012). The
63 approach allowed to detect very small percentages of adulterating oil; indeed, ~~, because~~ seed oils
64 have a concentration of phospholipids which is 300-400 times higher than olive oil (Koidis &
65 Boskou, 2006), thus determining a noticeable difference in the lipidomic profile. In order to exploit
66 the phospholipid fingerprint of olive oil in authenticity evaluations, an efficient and selective
67 extraction method as well as a suitable instrumental technique are needed. In most cases, the Folch

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68 (Folch, Lees, & Sloane Stanley, 1957) or Bligh and Dyers (Bligh & Dyer, 1959) methods are used
69 for sample pretreatment prior to phospholipid ~~analyses~~ determination, followed by clean-up or
70 enrichment. Subsequent analysis is usually performed by high performance liquid chromatography
71 (HPLC) coupled to high- or low-resolution mass spectrometry (MS). Matrix effect (ME) is a major
72 issue when HPLC-MS analysis of phospholipids is performed, thus making the clean-up/enrichment
73 stage fundamental to avoid the ionic suppression by the more concentrated species found in oil
74 (mostly triacylglycerols). A range of procedures have been developed for phospholipid enrichment,
75 including ~~several~~ solid phase extraction (SPE) strategies, in various matrices(Wang, Wang, & Han,
76 2015; Wei et al., 2018). As for olive oil, ~~D~~diol and silica cartridges have been compared by Verardo
77 et al. (Verardo et al., 2013), which identified 13 phospholipids by HPLC- quadrupole-time of flight
78 (TOF) MS analysis. A procedure based on amino-propyl SPE, followed by hydrophilic interaction
79 liquid chromatography coupled to ion trap MS, allowed to identify 18 species (Alves et al., 2016).
80 Also, matrix assisted laser desorption ionization (MALDI) coupled to TOF MS has been exploited
81 for phospholipid profiling in olive oil: in the work by Calvano et al. (Calvano et al., 2012), ionic
82 liquids were used for sample treatment, but only 4 phospholipids were tentatively identified. On the
83 other hand, an efficient graphene/TiO_2 matrix assisted solid phase dispersion technique permitted a
84 selective extraction, with subsequent identification of a total of 37 phospholipid species by MALDI-
85 TOF (Shen et al., 2013), with a prevalence of PC, PE and PI species.
86 The detection of a relatively limited number of phospholipids in the olive oil matrix could be
87 ascribed to insufficient clean-up from interferent species, incomplete recovery of the desired
88 analytes during sample preparation or low sensitivity, which hinder the identification of the less
89 concentrated compounds.
90 In this framework, the present paper deals with the comparison of two different solid phases, weak
91 anion exchange (WAX) and graphitized carbon black (GCB), for the enrichment of phospholipids
92 from olive oil. The WAX phase, constituted by aromatic moieties and charged piperazine units, is
93 able to create both ionic and lipophilic interactions (Marshall, Adaway, & Keevil, 2018); on the

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94 other hand, GCB possesses lipophilic characteristics and positively charge binding sites, under
95 acidic conditions (Capriotti et al., 2015). Their chemical composition made them theoretically
96 suitable to provide the enrichment of phospholipids in the olive oil. ~~The~~ Both procedures were
97 optimized in terms of recovery of some reference standards and the best procedure was applied to
98 olive oil phospholipidome determination. ~~and analyses~~ Analyses were performed by Ultra HPLC
99 (UHPLC) in combination with high resolution MS (HRMS). ~~I~~ and identification of phospholipids
100 was achieved using the recently developed software Lipostar (Goracci et al., 2017), by exploiting a
101 proper database for the considered class. A validation of the optimal methodology was performed
102 for some representative phospholipids, for which absolute quantitative data were provided. The
103 proposed method aims to provide a wide coverage in the identification of phospholipid species in
104 olive oil, from low concentration to trace levels, thanks to an enrichment-based approach, which, to
105 the best of our knowledge, has never been reported so far.

106

107 **2. Materials and methods**

108 **2.1 Chemicals**

109 Ultra-pure water of LC-MS grade was supplied by Thermo Fisher Scientific (Waltham,
110 Massachusetts, U.S.A.). Ultra-pure LC-MS methanol (MeOH) was obtained from Romil Pure
111 Chemistry (Pozzuoli, NA, Italy). Ammonium formate, formic acid, hydrochloric acid and
112 tetramethylammonium chloride (TMACl) were purchased from Sigma (St. Louis, MO, U.S.A.).
113 HPLC-grade chloroform, MeOH and water used for sample preparation were provided by VWR
114 International (Milan, Italy). Dichloromethane (DCM) and ethanol (EtOH) were obtained from Carlo
115 Erba Reagents (Milan, Italy). The standards 1,2-dilauroyl-sn-glycero-3-phosphocholine
116 [PC(12:0/12:0)], 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
117 [PG(14:0/14:0)], 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine [PE (18:1/18:1) (Δ9-Cis)] and 1-
118 stearoyl-2-heptadecanoyl-sn-glycero-3-phospho-L-serine (sodium salt) [PS (18:1/17:0)] were
119 purchased from Avanti Polar Lipids (Alabama, U.S.A.); the standard 1-palmitoyl-2-linoleoyl-sn-

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120 glicero-3-phosphate (sodium salt) (PA 16:0/18:2(9Z,12Z)) was purchased from Sigma (St. Louis,
121 MO, U.S.A). Stock standard solutions of single phospholipids were prepared in $CHCl_3$ at 1 mg L^{-1}
122 concentration and stored at -80 °C. Working solutions containing all compounds were prepared by
123 proper dilutions of the stock standards in $MeOH/H_2O/CHCl_3$, 60:35:5 (*v*/*v*), a composition matching
124 the initial composition of the LC-HRMS analysis gradient.
125

126 **2.2 Instrumentation**

127 The UHPLC system was a Vanquish chromatograph (Thermo Fisher Scientific) equipped with a
128 binary pump, a thermostated column compartment and an autosampler (kept at 14 °C). The
129 chromatographic column was a Kinetex EVO 100 × 2.1 mm, 1.7 μm particle size (Phenomenex,
130 Torrance, CA, U.S.A.) and the UHPLC conditions were those optimized for lipid analysis in our
131 previous work (La Barbera et al., 2018). Phase A and phase B were water and MeOH, respectively,
132 both with the addition of 5 mmol L^{-1}ammonium formate. The following gradient was employed:
133 from the initial condition of 60% B, B was increased to 70% from 0.1 to 5 min, further increased to
134 99% from 5.1 to 30 min, and kept constant for 5 min; finally, B was reported to 60% and the
135 column was allowed to equilibrate for 10 min (total analysis time 45 min). A flow rate of 0.4 mL
136 min^{-1} and a temperature of 40 °C were employed. The injection volume was 20 μL. Instrumental
137 analyses were performed in triplicate and two injections of a blank sample ($MeOH/H_2O$, 60:40 *v*/*v*)
138 were performed before each analyses batch to allow column conditioning and subtract blank sample
139 in data analysis.
140 The UHPLC system was coupled to a Q Exactive^{TM} hybrid quadrupole-Orbitrap mass spectrometer
141 (Thermo Fisher Scientific). A heated electrospray ionization (HESI) source, operating in negative
142 polarity mode, was used and the following tune parameters were set for the acquisition: capillary
143 temperature 320 °C; sheath gas 35 arbitrary units (a.u.); auxiliary gas 15 a.u.; spray voltage 2.5 kV;
144 auxiliary gas heater temperature 400 °C; S-lens RF level 100%. Spectra were acquired in top 5 data
145 dependent mode. For full-scan spectra acquisition, the resolving power was set at 140,000 (full

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146 width at half-maximum, FWHM, at *m/z* 200), scan range 200−1200 *m/z*, automatic gain control
147 (AGC) at 5 $\times 10^5$, maximum ion injection time at 200 ms, and isolation window width of 2 *m/z*.
148 Tandem MS fragmentation was obtained by higher-energy collisional dissociation at 40%
149 normalized collision energy, with a resolution of 70,000 (FWHM, at *m/z* 200), ACG 5×10^5 and
150 dynamic exclusion of 6 s. External calibration of the mass spectrometer was carried out every 2
151 days, within a mass accuracy of 1 ppm, using the commercial Pierce positive and negative
152 calibration solutions (Thermo Fisher Scientific). Raw MS/MS data files were acquired by Xcalibur
153 software (version 3.1, Thermo Fisher Scientific).
154

155 **2.3 Liquid-liquid extraction**

156 Extra virgin olive oil, from the Italian region Lazio, was obtained from a local supplier. The sample
157 was kept in the dark at room temperature; refrigeration was avoided to prevent any crystallization or
158 formation of precipitate which could incorporate phospholipids (Verardo et al., 2013). The olive oil
159 sample was shaken and sonicated before each enrichment experiment to ensure sample
160 homogeneity. Each sample preparation was performed in triplicate in the same day. The extraction
161 protocol was that proposed by Galanos and Kapoulas (Galanos & Kapoulas, 1962) with few
162 modifications. One gram of oil was dissolved in 4 mL of hexane and 1 mL of EtOH/H_2O, 80:20
163 (*v*/*v*) was added to the sample, vortex shaken for 15 min and put in an ultrasonic bath (Stitmin,
164 Milan, Italy) at 25 °C for 15 min. The sample was then centrifuged at 130 × g for 15 min; the
165 hydroalcoholic fraction containing phospholipids was collected and the procedure was repeated two
166 more times. For the WAX-SPE, the pooled extracts (3 mL) were dried down and the residue
167 reconstituted with 10 mL of hexane. For the GCB-SPE, the 3 mL extract was diluted with 9 mL
168 H_2O and pure TFA was added to obtain a solvent composition of EtOH/H_2O, 20:80 (*v*/*v*) 20 mmol
169 L^{-1} TFA.
170

171 **2.4 WAX-solid phase extraction**

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WAX SPE cartridges containing 500 mg of sorbent were purchased from Waters (Dublin, Ireland). The cartridge was first flushed with 15 mL of MeOH and then conditioned with 5 mL of hexane. After loading the sample, the cartridge was washed with 5 mL of hexane and analytes eluted by 10 mL of 0.65 mol L^{-1} TFA in DCM/MeOH, 95:5 (*v*/*v*) and 5 mL of 1.3 mol L^{-1} TFA in DCM/MeOH, 80:20 (*v*/*v*). Before UHPLC-HRMS analysis, the eluate was reduced to 100 µL by a rotary evaporator (mod. IKA RV 8, IKA-Werke GmbH & Co. KG, Staufen, Germany) and made up to a 200 µL volume to obtain a final solvent composition of ~~H_2O/MeOH/$CHCl_3$~~ MeOH/H_2O/$CHCl_3$, 80:15:5 (*v*/*v*/*v*).

2.5 Enrichment of phospholipids by GCB-solid phase extraction

The SPE cartridges containing 250 mg of GCB were purchased from LARA (Rome, Italy). In particular, the phase was Carbograph-4, which is a GCB with surface area of 210 m^2 g^{-1} and particle size in the range 120–400 mesh. The cartridge was conditioned by sequentially flushing 5 mL of 20 mmol L^{-1} TFA in DCM/MeOH, 80:20 (*v*/*v*), 5 mL of 20 mmol L^{-1} TFA in MeOH, 10 mL of 100 mmol L^{-1} HCl in H_2O and 10 mL of 20 mmol L^{-1} TFA in H_2O/EtOH, 80:20 (*v*/*v*); afterwards, the sample was loaded and the cartridge washed with 5 mL of 20 mmol L^{-1} TFA in H_2O/EtOH, 80:20 (*v*/*v*). A small volume of MeOH (500 µL) was used to remove any trace of water from the cartridge. Finally, the elution step was carried out in back-flushing mode: a Teflon piston with a Luer tip was inserted into the SPE cartridge, the cartridge was turned upside-down and an empty tube was positioned on top of it, to allow the solvent addition. Elution was performed by 10 mL of 20 mmol L^{-1} TFA in DCM/MeOH, 80:20 (*v*/*v*). Before UHPLC-HRMS analysis, the eluate was reduced to 100 µL by a rotary evaporator and diluted to 200 µL to obtain a final solvent composition of ~~H_2O/MeOH/$CHCl_3$~~MeOH/H_2O/$CHCl_3$, 80:15:5 (*v*/*v*/*v*).

2.6 Lipid identification

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197 Lipids identification was achieved by using the up-to-date software for lipidomics Lipostar. First,
198 the Lipostar DB Manager utility was used to build a customized database with exact masses and
199 theoretical MS/MS spectra of a large number of phospholipids. More specifically, by means of the
200 Lipid Builder Tool, lipid polar heads (i.e. phosphocholine, phosphatidic acid,
201 phosphoethanolamine, phosphoglycerol or phosphoserine heads) were combined with either 1
202 (lyso-forms) or 2 acyl chains ranging from C3 to C35 length and from 0 to 9 double bonds. In the
203 case of sphingomyelins (SM), the phosphocholine, phosphoethanolamine and phosphoinositol
204 heads were combined with a ceramide group, in turn bound to an acyl chain with length from C3 to
205 C35 and 0-9 double bonds. All these combinations were used to obtain a database of exact masses
206 and associated chemical formulas. Then, common fragmentation rules were applied to create the
207 MS/MS spectra and to select specific product ions as class- and compound-diagnostic. For the
208 lipidomic profiling, accurate mass ion chromatograms, obtained by the UHPLC-HRMS analysis,
209 were processed by Lipostar and identification of phospholipids was possible by the implementation
210 of the described database. A pre-processing of the chromatograms was carried out by applying
211 baseline and noise reduction, peak extraction, smoothing, signal-to-noise ratio filtering, deisotoping
212 and deconvolution, according to the parameters reported in Table S1. Afterwards, database search
213 of the experimental MS and MS/MS spectra was carried out on the raw data file for lipid
214 identification, by specifying a tolerance of 5 ppm and 10 ppm for the precursor and the product ion
215 mass, respectively. Lipid identifications based on both precursor ion masses and MS/MS fragments
216 were manually checked to eliminate false positives.
217

218 **2.7 Analytical method performances**

219 The developed analytical method was evaluated in terms of the following characteristics: linearity
220 range, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and ME. These
221 evaluations were carried out for five representative standards of the phospholipid classes, namely
222 PA (16:0/18:2), PE (18:1/18:1), PS (18:1/17:0), PG (14:0/14:0) and PC (12:0/12:0). External

223 calibration curves were built by analysing neat standards in solvent, at 5 concentration levels in the
224 range 0.1-1 ng μL $^{-1}$ for PC (12:0/12:0), PG (14:0/14:0) and PE (18:1/18:1) and 0.3-6 ng μL $^{-1}$ for
225 PA (16:0/18:2) and PS (18:1/17:0). Higher concentrations were selected for PA (16:0/18:2) and PS
226 (18:1/17:0) due to the lower sensitivity for these compounds. Matrix-matched calibration curves
227 were obtained by spiking oil samples at the same concentrations previously used for the external
228 calibration curves in solvent, and the dynamic linear ranges were verified. Recovery of the GCB-
229 SPE procedure was assessed by six replicate extractions on spiked oil sample. Two fortification
230 levels were considered: the low level was 5 μg mL^{-1} for PC (12:0/12:0), PG (14:0/14:0) and PE
231 (18:1/18:1), 20 μg mL^{-1} for PS (18:1/17:0) and 10 μg mL^{-1} for PA (16:0/18:2); the high fortification
232 level was 10 μg mL^{-1} for PC (12:0/12:0), PG (14:0/14:0) and PE (18:1/18:1), 40 μg mL^{-1} for PS
233 (18:1/17:0) and 20 μg mL^{-1} for PA (16:0/18:2). The recovery study was exploited to assess the
234 method accuracy; in fact, phospholipids are endogenous compounds in olive oil and no certified
235 reference material was available. Precision was estimated by performing intra-day (n=6) and inter-
236 day (n=6, in 6 non-consecutive days) repeatability assays, involving the whole pre-treatment and
237 analysis workflow. LOD and LOQ values were estimated by the following approach (Kruve et al.,
238 2015): a value of $3\times\delta q/m$ and $10\times\delta q/m$ were taken as the method LOD and LOQ, respectively,
239 where q is the intercept of the matrix-matched calibration curve, δq is its standard deviation and m
240 is the slope of the curve. Solutions at these concentrations were injected for confirmation. ME (ion
241 suppression or enhancement) was calculated by comparing the chromatographic signal of the
242 analytes in neat standard solutions and in the oil extract, at the same concentrations (Matuszewski,
243 Constanzer, & Chavez-Eng, 2003). Furthermore, ME was evaluated by comparing the external and
244 matrix-matched calibration curves, in terms of sensitivity (curve slope) and linearity range.In detail,
245 the presence of ME was verified by using the following equation:

246
$$ME\ (\%) = 100 * \frac{A_A - A_{NS}}{A_P}$$

247 where A_A, A_{NS} and A_P are the chromatographic peak areas obtained by analyzing samples spiked
248 after the GCB SPE (A), non-spiked samples (NS) and the pure standard solution (P), respectively.

249 Moreover, the slopes of the matrix-matched and external calibration curves were compared by
250 calculating the m_{mm}/m_{ec} ratio, where m_{mm} is the matrix-matched curve slope, and m_{ec} is the external
251 curve slope.
252

253 **3. Results and discussion**

254 **3.1 Development of the enrichment procedure: WAX vs GCB**

255 Phospholipids are minor constituent in olive oil and their detection is hindered by the presence of
256 other abundant lipid species, being this matrix composed mainly of triacylglycerols. Therefore, to
257 develop a specific method for phospholipid identification, a careful study of the sample pre-
258 treatment was necessary. A simple SPE strategy was selected after liquid-liquid extraction of the
259 olive oil sample. Two different solid phases were tested, selected on the basis of phospholipid
260 chemical structures, i.e. WAX and GCB. WAX is a mixed-mode sorbent, able to provide both
261 hydrophilic and lipophilic interactions, as well as presenting binding sites with weak basic
262 properties. Indeed, this solid phase is constituted by silica functionalized with chains containing
263 aromatic groups and piperazine; these molecular units could interact with the phospholipids via
264 lipophilic interactions with the fatty acid chains and ionic interactions with the phospholipids polar
265 head, due to the positive charge of the piperazine nitrogen atoms, established in acidic ambient.
266 Instead, GCB is a versatile phase, composed of a graphite-type structure, mainly possessing
267 lipophilic characteristics, and positively charged binding sites, due to the presence of impurities,
268 which are incorporated during the preparation of the material. These sites are chromene-like groups
269 which need to be activated by reaction with strong acids (Andreolini, Borra, Caccamo, Di Corcia, &
270 Samperi, 1987). The phospholipid polar heads are characterized by the presence of negatively
271 charged groups, thus rendering the described phases suitable for their isolation from a complex
272 matrix and providing a clean-up from neutral lipids as well as other neutral or basic compounds
273 (Sato, Nakamura, Aoshima, & Oda, 2010). In order to maximize phospholipid recovery of the SPE
274 procedures, a standard mix of five phospholipids, representative of the main subclasses, was

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275 prepared in loading solvents. The selected concentrations (10-40 ng μL^{-1}, approximately
276 corresponding to 10-40 mg kg^{-1}) were slightly higher than the expected value in the considered
277 matrix, in the order of mg kg^{-1}, to test the loading capacity of the cartridges (Hatzakis, Koidis,
278 Boskou, & Dais, 2008). Different loading and eluting solvents were tested for the two SPE
279 procedures, based on the literature data or manufacturer instructions, with some modifications, and
280 the efficiency of the retention and elution mechanisms were verified. In particular, for the WAX
281 procedure, hexane was selected as the loading solvent, being the only suitable solvent for the
282 interaction of polar lipids with the WAX solid phase; as far as elution is concerned, MeOH and
283 DCM in different proportion and TFA concentration were tested. Two subsequent elutions with
284 increasing acid concentration resulted as the best option: 10 mL of 0.65 mol L^{-1} TFA in
285 DCM/MeOH, 95:5 (*v*/*v*) followed by 5 mL of 1.3 mol L^{-1} TFA in DCM/MeOH, 80:20 (*v*/*v*) were
286 used to elute phospholipids. These conditions allowed retention and satisfactory recovery (50-
287 120%) of the standard compounds. For GCB, the starting point was a procedure developed in our
288 lab for other polar acidic substances (data not published); phospholipids are insoluble in pure water,
289 therefore, although the most suitable loading solvent for GCB SPE is water, the minimum
290 percentage of EtOH necessary to guarantee the analytes solubility was maintained, resulting in a
291 loading mixture of EtOH/H_2O, 20:80 (*v*/*v*). The tested elution solvent mix was DCM/MeOH, 80:20
292 (*v*/*v*) with the addition of TMACl or TFA at different concentrations, reaching the highest recovery
293 with 20 mmol L^{-1} TFA (60-90%). The developed strategies were then tested on an oil sample,
294 spiked before liquid-liquid extraction. Figure 1 shows the average recoveries obtained from all the
295 replicate tests on the spiked sample. Although comparable performances of the two solid phases
296 were observed, thus far on the simple standard mix, recoveries from oil were rather poor when
297 WAX was used, with values below 65% for all standards (2-65%). This behaviour could be
298 ascribed to the formation of reverse micelles in hexane (Pérez-Cejuela et al., 2018); in fact, when
299 the critical micelle concentration (CMC) is reached in non-aqueous systems the phospholipids polar
300 head disposes toward the inner part of the micelle, thus potentially hampering the interaction with

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301 the ionic exchange sites on the WAX surface. It is possible that the phospholipid concentration in
302 the real spiked sample was above the CMC, resulting in insufficient retention. On the other hand,
303 the GCB protocol led to satisfactory results, demonstrating a high capacity and specificity of the
304 phase with respect to the considered analyte class, despite the complexity of the olive oil matrix,
305 and allowing to obtain a phospholipid recovery ranging from 63 to 101%. In addition to the
306 quantitative evaluation, the two SPE procedures were compared on the basis of the number of
307 identified phospholipids in the final eluates. A total of 82 phospholipids, belonging to seven
308 subclasses, were recognized by analysing the GCB eluate, while only 32 phospholipids were
309 detected when WAX SPE was performed, probably due to both low recovery and inadequate
310 purification. These results confirmed that the GCB enrichment allowed a high recovery of the
311 considered classes and an efficient clean-up, which limits ion suppression of the less abundant
312 species, thus leading to a ~~4-fold larger number~~ larger number of identifications.
313

314 ### 3.2 Phospholipids identification in the enriched extracts

315 The main purpose of the present work was to obtain a high coverage in phospholipids identification,
316 to provide the best possible qualitative and semi-quantitative profile of olive oil. To this aim, the
317 samples deriving from the two enrichment methods (WAX and GCB) were compared in terms of
318 number of phospholipids identified by using the Lipostar software. Lipostar (Goracci et al., 2017)
319 currently constitutes the most exhaustive software suited for lipidomic analysis in complex
320 matrices, for both untargeted and semi-targeted approaches. Exact masses of single phospholipids
321 and MS/MS spectra, included in the homemade database, were used in combination to validate the
322 identified compounds. In fact, diagnostic product ions referring to lipid subclasses or specific acyl
323 chains composition were included in Lipostar DB manager as mandatory or recommended ions,
324 allowing to avoid false-positives and to achieve a higher identification confidence level (Goracci et
325 al., 2017). In the Lipostar software, the confidence level of lipid identification is provided by a
326 stars-based classification system: four stars are assigned if both mandatory and recommended

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327 product ions are recognized in the MS/MS spectra, including at least one fragment of a fatty acid
328 chain; three stars are provided if at least one mandatory product ion is identified; instead, if at least
329 one recommended product ion is identified, stars drop to two. Finally, one star is scored if neither
330 mandatory nor recommended product ions are identified, and the assignment is only based on the
331 exact mass. The knowledge of the fragmentation behaviour of phospholipids and sphingomyelins,
332 already studied in the literature (Chen et al., 2017; Han & Gross, 1996; F. Hsu & Turk, 2009; Pulfer
333 & Murphy, 2003), was exploited to select recommended and mandatory ions of precursor lipid
334 species, which were included in the Lipostar DB Manager. Based on fragmentation rules, the
335 product ions $[R_1CO_2]^-$ and $[R_2CO_2]^-$, corresponding to the loss of a fatty acid chain as carboxylate
336 anion, have been assigned as mandatory for all phospholipid classes (PC, PG, PA, PS, PE, PI and
337 the related lyso-forms). In fact, these fragments are usually the base peaks in all MS/MS spectra,
338 with the cleavage of the C-O bond in the *sn2* position of the glycerol backbone being the most
339 probable (Hsu & Turk, 2001). The product ion at *m/z* 152.9958 ($[C_3H_6O_5P]^-$), which corresponds to
340 the phosphoglycerol group, has been introduced as mandatory for PG, PA, PS and their
341 corresponding lyso-forms (Chen et al., 2017); it results from the elimination of the acyl chains
342 along with glycerol and serine group from the precursor $[M-H]^-$ and subsequent loss of H_2O. In
343 addition, the fragment at *m/z* 241.0118 ($[C_6H_{10}O_8P]^-$), namely the inositol-phosphate group, has
344 been selected as mandatory for PI and LPI. Finally, the mandatory fragments for SM were the ion at
345 *m/z* 78.9590, corresponding to $[PO_3]^-$ and the ion at *m/z* 168.0431 ($[C_4H_{11}NO_4P]^-$), matching the
346 phosphocholine head, for the sphingomyelin-phosphocholine class. Concerning recommended
347 product ions, the fragments at *m/z* 78.9590 ($[PO_3]^-$) and at *m/z* 96.9696 ($[H_2PO_4]^-$) were assigned as
348 common to all phospholipid classes.
349 Additionally, specific ions for each lipid class, either related to the polar head groups or to its
350 structure rearrangements, were set as recommended. A detailed discussion of the recommended
351 product ions of each class is presented in the supplementary material. Furthermore, as exhaustively
352 discussed in our previous work (La Barbera et al., 2018), the false positive identifications due to the

353 in-source fragmentation of PC and PS were eliminated. Following the described rules, the data
354 processing led to the identification of 32 and 82 phospholipids in the olive oil WAX-eluate and
355 GCB-eluate, respectively. These results were obtained by keeping only the compounds to which 3
356 or 4 stars were assigned as confidence level, and that were manually validated. Tables 1 and 2
357 display the representative lipid subclasses identified with corresponding fragments grouped in
358 product ions common to phospholipids, common to a specific class and depending on the fatty acid
359 composition; the confidence level was assigned according to the literature (Schymanski et al.,
360 2014). Briefly, the confidence levels were 1, 2a, 2b and 3 according to the identified product ions;
361 in particular, 2a was assigned to the lipids for which the fatty acid chains position was determined,
362 based on the intensity of the fragments (Hsu & Turk, 2009). In details, the most abundant and the
363 less abundant fatty acid chain fragments, as carboxylate anions, correspond to the loss in sn-2 and
364 sn-1 position on the glycerol backbone, respectively. Hence, the position of fatty acid chains could
365 be assigned. Moreover, in the case of lyso-forms, level 2b was assigned also when the fatty acid
366 fragment was missing because the length of the acyl chain could be deduced from the phospholipid
367 exact mass. Furthermore, the entire list of identified lipids, both with GCB and WAX enrichment
368 procedures, are presented in Tables S2 and S3. Figure 2 shows the number of phospholipids
369 belonging to each subclass for the two sample pre-treatments. In the sample treated by WAX-SPE,
370 the number of identified lipids belonging to the different classes was homogeneous (from 1 to a
371 maximum 6 phospholipids per class) and always lower if compared with the other procedure. In
372 particular, GCB-SPE allowed a better enrichment of PA and PG over the other species (22 and 30
373 identifications, respectively). This result could be ascribed to a particular selectivity of the GCB
374 material towards these two classes as well as their higher concentrations in the olive oil sample.
375 Lyso-phospholipids were the sole compounds that were found in higher number in the case of
376 WAX-SPE; only 2 LPS were identified in the GCB-eluate, while 1 LPS, 3 LPE, 5 LPA and 1 LPG
377 were identified in the WAX-eluate. The final elution procedure for the WAX-SPE was performed
378 with a higher concentration of TFA compared to the GCB-SPE. Under such acidic condition, the

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379 phospholipid lyso-forms could be generated as hydrolytic artefacts (Sato et al., 2010) leading to an
380 improper identification. Finally, the qualitative profiles of the two SPE eluates were compared in
381 terms of the common lipids. The two SPE procedures provided different lipid profiles:, as only the
382 most abundant and widespread phospholipids in plants, namely LPS(18:0), PA(14:0/14:0),
383 PC(18:1/18:1), PA(18:1/18:1), PG(14:0/14:0) and PE(18:1/18:1) (Millar, Smith, & Kunst, 2000),
384 were enriched by both systems.
385 Regarding a comparison with literature data, the most detailed phospholipid profile of olives and
386 olive oil was depicted by Shen et al (Shen et al., 2013). Compared to this work, where 37
387 phospholipids were identified, with a majority of PC, our optimized GCB-SPE procedure combined
388 with the Lipostar platform led to a definitely higher number of identifications (82 species), with a
389 larger number of PA and PG species. Moreover, two additional lipid classes, namely PS and SM
390 were identified with our analytical method.
391
392
393 **3.3 Method performances**
394 The final method is mainly aimed at qualitative analysis and determination of relative
395 concentrations of the single identified phospholipids; nevertheless, absolute quantitation of selected
396 phospholipids was accomplished. For this purpose, several criteria were considered to validate the
397 methodology for five phospholipids, representative of different subclasses, namely PC(12:0/12:0),
398 PG(14:0/14:0), PA(16:0/18:2), PE(18:1/18:1) and PS(18:1/17:0). Table 3 summarizes the method
399 performance. Some preliminary tests were performed to roughly estimate the expected
400 concentrations of the selected compounds and therefore choose the range for calibration. Linearity
401 was then tested by building both external and matrix-matched calibration curves; the complexity of
402 the matrix slightly reduced the linear range for all compounds, except for PA(16:0/18:2), compared
403 to the external calibration. As already mentioned, recoveries of the GCB-SPE procedure were
404 satisfying for both high and low fortification levels, with mean values ranging from 63 to 101%.

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405 The intra-day repeatability, evaluated as RSD% of six replicate determinations performed in a
406 single day, was <10% for all analytes, except for PA(16:0/18:2), which exhibited a slightly larger
407 value (12%); as far as the intermediate precision is concerned (inter-day assay), the RSD values
408 were always < 20% ($n=6$). Both recovery and precision parameters complied with the acceptance
409 criteria of validation method guidelines ("European Commission Decision 2002/657/EC
410 Implementing Council Directive 96/23/EC concerning the performance of analytical methods and
411 the interpretation of results," 2002; "Guidance document on analytical quality control and
412 validation procedures for pesticide residues analysis in food and feed," 2014). Thanks to the sample
413 purification as well as the powerful UHPLC-MS analysis, high sensitivity was reached, with LODs
414 and LOQs in the range of 9.2-36 ng g^{-1} and 30.5-120 ng g^{-1} respectively. These values are definitely
415 lower than the ones found in the literature, usually at concentration levels of µg g^{-1} (Montealegre,
416 Sánchez-Hernández, Crego, & Marina, 2013; Verardo et al., 2013).~~The presence of ME related to~~
417 ~~ion suppression or enhancement was verified for the five standards by using the following equation:~~

418 $$ME\ (\%) = 100 * \frac{A_A - A_{NS}}{A_P}$$

419 ~~where A_A, A_{NS} and A_P are the chromatographic peak areas obtained by analyzing samples spiked~~
420 ~~after the GCB SPE (A), non-spiked samples (NS) and the pure standard solution (P), respectively.~~
421 ~~Moreover, the slopes of the matrix-matched and external calibration curves were compared by~~
422 ~~calculating the m_{mm}/m_{ec} ratio, where m_{mm} is the matrix-matched curve slope, and m_{ec} is the external~~
423 ~~curve slope.~~Two criteria, mentioned in the section 2.7 "Analytical method performances", were
424 used to calculate the matrix effect. Both ~~Both criteria~~ were in accordance and demonstrated that ion
425 suppression was not negligible for these analytes, being ME% in the range 20-50%. For this reason,
426 the standard addition method, by using matrix-matched calibration curves, was taken as the most
427 suitable for quantitation and used accordingly.

428

429 **3.4 Semi-quantitative analysis**

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430 The GCB enrichment method allowed to obtain a wide coverage of phospholipids, belonging to
431 several subclasses and present at different concentration levels in the olive oil sample. Given the
432 validation parameters obtained for the five representative phospholipids, we could assume that good
433 recovery and sensitivity was reached for species belonging to the same classes; nonetheless, ME
434 probably affected the intensity of the chromatographic peaks. Therefore, only a relative "semi-
435 quantitative" analysis could be performed for the phospholipids identified in untargeted fashion.
436 The chromatographic peak area of the identified lipids was integrated by Lipostar software and used
437 to calculate the relative abundances; the pie-chart in Figure 3 shows the quantitation results by
438 indicating the abundance of the different phospholipid classes detected in the extra virgin olive oil
439 sample. The percentages of each identified phospholipid over the total are reported in Table S4. The
440 most abundant class was represented by PAs, which constituted the 59% of the total phospholipids,
441 followed by PGs (16%), while PS, PI, PC and PE were all under the 10%. These results are in
442 accordance with Hatzakis et al. (Hatzakis et al., 2008), which analyzed phospholipids in olive oil by
443 ^{31}P NMR, detecting PA species as the dominant, while PI and PC representing only a small portion.
444 Likewise, Verardo et al. (Verardo et al., 2013) reported PA as the major class, representing the 60%
445 of total phospholipids. Other papers reported PGs species as the most abundant, in partial agreement
446 with our results (Boukhchina, Sebai, Cherif, Kallel, & Mayer, 2004; Calvano et al., 2012). Finally,
447 it is noteworthy that some works reported the PC class as the most numerous, when using positive
448 ionization mode (Alves et al., 2016; Shen et al., 2013), which induces a more efficient ionization for
449 these species (Hsu & Turk, 2009). Nevertheless, we selected the negative mode to obtain a more
450 reliable identification, thanks to formation of the acyl chains fragments, which allows to confirm the
451 phospholipids identity.
452 Alongside with the relative quantitation, an absolute quantitative analysis was possible for the five
453 representative standards, by applying the standard addition method. PC(12:0/12:0), PG(14:0/14:0)
454 and PA(16:0/18:2) were detected at comparable concentrations, i.e. 43 ± 3 ng g^{-1}, 35 ± 3 ng g^{-1} and
455 137 ± 17 ng g^{-1}, respectively; on the other hand, PS(18:1/17:0) and PE(18:1/18:1) were below the

456 method LOQ. These results provide an estimation of the concentration levels of single
457 phospholipids and demonstrate that an enrichment is crucial in the identification of low abundant
458 species.
459

460 **4. Conclusions**

461 The present work compared two different SPE materials, WAX and GCB, for the enrichment of
462 phospholipids from extra virgin olive oil. The procedures, tested and optimized on some reference
463 standards, were used to define the extra virgin olive oil polar lipidome. The analyses by UHPLC-
464 HRMS, which provides accurate MS and MS/MS data, were performed in negative polarity mode,
465 which is the most suitable ionization mode to identify phospholipids and their corresponding acyl
466 chains. On this basis, identification of phospholipids was achieved by the recently developed
467 software Lipostar and the implementation of a customized database for phospholipids. GCB-SPE
468 revealed to be the best method and led to the identification of 82 phospholipids in olive oil, which
469 is, to the best of our knowledge, the highest number ever reported. Moreover, the developed
470 methodology, validated for some standards considering the classical figures of merit, provided
471 absolute quantitative data for PC(12:0/12:0), PG(14:0/14:0) and PA(16:0/18:2), found at
472 concentrations in the range 35-137 ng g^{-1}. On the other hand, the selected PE and PS standards were
473 below the method LOQ. These data can be considered as a starting point to estimate the
474 concentration of each subclass identified in the sample. Besides, the relative quantitation allowed us
475 to estimate the polar lipid profile of the olive oil sample, highlighting PAs and PGs as the most
476 abundant species. This work emphasizes the crucial importance of a SPE procedure to obtain a
477 higher number of identified lipids thanks to clean-up and enrichment, being phospholipids minor
478 components in the extra virgin olive oil matrix.
479

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486

487 **Conflict of interest**

488 The authors declare no conflict of interests

489

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Figure Captions

Fig. 1 Comparison of recoveries obtained for a spiked sample treated by WAX-SPE and GCB-SPE.

Fig. 2 Number of identified lipids belonging to the different phospholipids subclasses by applying the WAX-SPE and the GCB-SPE.

Fig. 3 ~~Relative quantitation~~ Semi-quantitation of phospholipids in the olive oil sample: percentages of the chromatographic peak areas over the total.

Highlights

- Enrichment of phospholipids in olive oil was performed.
- Weak anion exchange and graphitized carbon black were compared.
- 82 polar lipids were identified in olive oil by the bioinformatics tool Lipostar.
- Five phospholipid standards were quantified with detection limits at ng g^{-1} level.
- Phosphatidic acids and phosphatidylglycerols were the most abundant species.

Table 1: Representative lipid subclasses identified in the extra virgin olive oil sample with the GCB enrichment. (For the entire list of the identified lipids refer to Table S2)

...sured mass	**Δppm**	**Molecular formula**	**Adduct**	**Product ions common to phospholipids**	**Product ions common to one class of phospholipids**	**Product ions depending on fatty acid composition**	**Identity**
.3148	0.2	$C_{26}H_{51}O_{10}P$	$[M-H]^-$	78.9577; 96.9684; 152.9948		255.2327	PG(16:0/4:0)
.3872	0.5	$C_{32}H_{59}O_8P$	$[M-H]^-$	96.9589		255.2328	PA(13:2/16:0)
.4345	1.0	$C_{32}H_{64}NO_8P$	$[M+HCOO]^-$	78.9577;	168.0423; 24.0688	199.1696;	PC(12:0/12:0)
.4396	0.5	$C_{34}H_{67}O_{10}P$	$[M-H]^-$	78.9577; 96.9590; 152.9947	171.0054	227.201	PG(14:0/14:0)
.4943	3	$C_{39}H_{71}N_2O_6P$	$[M-H]^-$	78.9577	168.0431		SM(d34:5)*
.5283	1	$C_{42}H_{78}NO_{10}P$	$[M-H]^-$	78.9577; 96.9683; 152.9948		281.2484	PS(18:1/18:1)
.4807	1	$C_{37}H_{71}O_8P$	$[M-H]^-$	78.9577; 96.9683; 152.9948		281.2484; 255.2326	PA(16:0/18:1)
.5443	0.5	$C_{42}H_{80}NO_{10}P$	$[M-H]^-$	78.9577; 96.9683; 152.9947		283.2640; 281.2484	PS(18:0/18:1)
5.6272	0.9	$C_{57}H_{93}O_{13}P$	$[M-H]^-$	78.9696; 152.9947	241.0116	281.2483	PI(18:1/30:8)

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.5759	0.			78.9598; 96.9682;			/18:0)
2.539	0.3	$C_{41}H_{78}NO_8P$	$[M-H]^-$	78.9577	140.0107; 196.0372	281.2484	PE(18:1/18:1)
7.7218	0.2	$C_{57}H_{105}O_{13}P$	$[M-H]^-$	78.9577	241.0116	281.2484	PI(18:1/30:2)

*Identified lipids for which it was not possible to discriminate the acyl chains: only the sum composition is indicated.

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Table 2: Representative lipid subclasses identified in the extra virgin olive oil sample with the WAX

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asured mass	Δppm	Molecular formula	Adduct	Product ions common to phospholipids	Product ions common to one class of phospholipids	Product ions depending on fatty acid composition	Identity
.2422	1.40	$C_{20}H_{41}O_9P$	$[M-H]^-$	78.9578; 152.9949	171.0056	227.2014	LPG(0:0/14:0)
.2474	1.00	$C_{19}H_{40}NO_7P$	$[M-H]^-$	78.9578; 152.9949	196.0375	227.2014	LPE(0:0/14:0)
.2365	1.20	$C_{19}H_{39}O_7P$	$[M-H]^-$	78.9578; 96.9684; 152.9949	171.0056	255.233	LPA(0:0/16:0)
.2997	0.60	$C_{24}H_{48}NO_9P$	$[M-H]^-$	78.9578; 96.9684; 152.9949			LPS(0:0/18:0)
.4276	4.10	$C_{35}H_{67}O_{13}P$	$[M-H]^-$		241.0116	73.0283	PI(23:0/3:0)
.4752	4.50	$C_{41}H_{73}O_{13}P$	$[M-H]^-$			281.249	PI(14:2/18:1)
.4359	1.10	$C_{32}H_{64}NO_8P$	$[M+HCOO]^-$	78.9579	168.0425	199.1700; 224.0693	PC(12:0/12:0)
.4404	0.70	$C_{34}H_{67}O_{10}P$	$[M-H]^-$	78.9578; 152.9949	171.0056	227.2013	PG(14:0/14:0)
.4457	0.60	$C_{33}H_{66}NO_8P$	$[M-H]^-$	78.9578	140.0108; 196.0375	227.2013	PE(14:0/14:0)
.5453	0.80	$C_{42}H_{80}NO_{10}P$	$[M-H]^-$	78.9578; 96.9684; 152.9949		281.2487; 283.2645	PS(18:0/18:1)
.5772	1.40	$C_{42}H_{82}NO_8P$	$[M+HCOO]^-$	78.9578	168.0425; 224.0693	255.2330; 281.2488	PC(16:0/18:1)
.5011	1.80	$C_{42}H_{75}O_{10}P$	$[M-H]^-$	78.9578; 96.9684; 152.9949		283.2644	PG(18:4/18:0)
.5401	1.20	$C_{41}H_{78}NO_8P$	$[M-H]^-$	78.9578	140.0108; 196.0375	281.2487	PE(18:1/18:1)

*Identified lipids for which it was not possible to discriminate the acyl chains: only the sum composition is indicated.

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Table 3: validation parameters of the GCB-UHPLC-HRMS method for selected phospholipids standards.

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External calibration		Matrix-matched calibration		LOD (ng g $^{-1}$)	LOQ (ng g $^{-1}$)	ME (%)	ME (m_{mm}/m_{ec})	RSD(%)[a] (Intra-day)	RSD(%)[b] (Inter-day)
Linearity range (ng µL^{-1})	R^2	Linearity range (ng µL^{-1})	R^2						
0.1-1	0.9988	0.18-0.7	0.9982	10.8	36.1	28.4	0.3	8.3	20
0.15-1	0.9963	0.15-0.8	0.9981	9.2	30.5	49.9	0.4	8.8	19.5
0.6-6	0.9935	0.6-6	0.9830	36.0	120.0	20.5	0.2	12.6	18.0
0.3-3.4	0.9997	0.57-3.4	0.9988	34.0	113.4	25.7	0.3	6.3	12.2
0.14-1	0.9989	0.37-0.8	0.9962	22.3	74.3	31.9	0.2	4.0	14.1

[a] relative standard deviation obtained from six replicate analyses performed in the same day

[b] relative standard deviation obtained from six replicate analyses performed in six non-consecutive days

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