



# New insights in hemp chemical composition: a comprehensive polar lipidome characterization by combining solid phase enrichment, high-resolution mass spectrometry, and cheminformatics

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## Abstract

The chemical composition of *Cannabis sativa* L. has been extensively investigated for several years; nevertheless, a detailed lipidome characterization is completely lacking in the literature. To achieve this goal, an extraction and enrichment procedure was developed for the characterization of phospholipids and sulfolipids. Firstly, a study on the solid-liquid extraction was performed, to maximize the recovery of the considered lipids; the best procedure consisted of a simple extraction with a mixture of methanol and chloroform (1:1, v/v). The hemp extracts were analyzed by ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry and lipids were tentatively identified by Lipostar. To improve the number of identifications, an enrichment method, based on graphitized carbon black solid phase extraction, was evaluated to fractionate phospholipids and sulfolipids into separate eluates. Recovery and matrix effects of the procedure were determined on a mixture of standard lipids, containing representative compounds for each considered lipid class. The optimized method allowed the tentative identification of 189 lipids, including 51 phospholipids and 80 sulfolipids, in the first and second fractions, respectively. The detection of only 6 sulfolipids in the first fraction and 9 phospholipids in the second fraction proved the efficacy of the fractionation method, which also allowed the number of lipid identifications to be increased compared to the same procedure without enrichment, which scored 100 lipids. Finally, a semi-quantitative analysis permitted the hemp polar lipidome to be characterized. The results of this study allow knowledge of the hemp chemical composition to be improved with a detailed description of its phospho- and sulfolipid profiles.

**Keywords** Lipidomics · Phospholipids · Sulfolipids · Graphitized carbon black · *Cannabis sativa* · Lipostar

## Introduction

*Cannabis sativa* L. has been well-known, since ancient times, for its multiple uses for medicinal, phytochemical, textile, and recreational purposes [1, 2]. It can be classified as drug-type cannabis or fiber-type cannabis (industrial hemp) depending on the concentration of the psychoactive compound delta-9-tetrahydrocannabinol (delta-9-THC), with a widely accepted cut-off of 0.3% (w/w) [3]. During the last decade, industrial hemp has been attracting increasing attention because of the various forms in which it can be consumed, e.g., as dried flower, oil, topicals, edibles, and beverages [4]. The establishment of the hemp market required a more exhaustive characterization of this complex matrix. Cannabis chemical composition is characterized by more than 400 chemical entities and cannabinoids represent more than 15% of the total [5]. Along with cannabinoids, several other constituents were detected

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and characterized in hemp. For example, terpenes are secondary metabolites responsible for the odor and flavor of the different cannabis strains [6] and were used as markers in *Cannabis* to establish the chemotaxonomy of the plant, along with cannabinoids [7]. Moreover, *Cannabis* fruits and roots contain phenolic compounds, including, for instance, cannflavin A and B, which exert a wide range of biological activities [8]. Phytocannabinoids, terpenes, phenolic compounds, and other minor components, such as fatty acids, amino acids, proteins, sugars, vitamins, and pigments, provide significant human benefits, due to both synergic and antagonist interactions [9]. In this context, the development of new extraction and enrichment methods to comprehensively characterize cannabis compounds represents a relevant scientific target with the final aim of discovering new compounds, especially related to the numerous bioactivities of this plant. The above-mentioned chemicals are commonly isolated from hemp inflorescence employing different solid-liquid extraction (SLE) methods [10], which could successfully be extended to the extraction of other species, thus providing a more in-depth matrix characterization.

Polar lipids represent a heterogeneous class of compounds, whose determination in plant matrices is raising the interest of the scientific community. Two main subclasses, namely phospholipids and sulfolipids, are the major constituents of vegetable cell membranes, with sulfolipids exclusively located in tylakoid membranes [11]. Phospholipids can be divided in phosphatidylcholines (PC), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI), phosphatidic acid (PA), lysophosphatidylcholines (LPC), lysophosphatidylinositols (LPI), lysophosphatidylglycerols (LPG), lysophosphatidylserines (LPS), lysophosphatidylethanolamines (LPE), sphingomyelins (SM), and lysophosphatidic acids (LPA); sulfolipids are classified in sulfoquinovosylmonoacylglycerols (SQDG) and sulfoquinovosyldiacylglycerols (SQMG), depending on the presence of one or two acyl chains on the sulfated glycerol backbone. A range of bioactivities has been attributed to these compounds, such as anti-inflammatory, antiatherosclerotic, and anti-carcinogenic properties [12, 13]. A recent review, concerning the biological activities of food phospholipids, emphasized the importance of the structure-activity relationship for human health: several properties, which depends on both the polar head and the fatty acid chain composition, were described (e.g., improvement of memory, antioxidant activity, and reduction of risk of cardiovascular diseases) [14]. The investigation of polar lipids in vegetable matrices usually involves SLEs and, possibly, clean-up strategies, followed by direct mass spectrometry (MS) or liquid chromatography-mass spectrometry (LC-MS) analysis [15]. Bligh and Dyer or Folch methods are the most commonly employed for liquid extractions, by using methanol-chloroform-water mixtures and consequent liquid-liquid repartition [16, 17]. Recently, some faster methods

involving a single-step SLE with mixtures of chloroform, methanol, isopropanol, and water were described [18, 19]. Solid phase extraction (SPE) can be used to provide cleaner extracts, especially if the analysis is focused on the determination of low-concentrated compounds. Silica cartridges, as well as ionic exchangers, were used for the purification of both sulfolipids and phospholipids [20, 21]. A recent work from our laboratory exploited the properties of graphitized carbon black (GCB) as solid phase for the enrichment of sulfolipids [22]; indeed, GCB possesses the capability of establishing several types of interactions (hydrophobic interactions,  $\pi$ - $\pi$  interactions, and electrostatic interactions), thus constituting a versatile phase, suitable to enrich compounds characterized by different chemical properties.

In this framework, this work describes a study on the extraction of the polar lipid fraction of hemp, to provide a wide characterization of its lipid profile and fill a gap in the current literature. SLE and SPE enrichments were optimized in order to obtain a large coverage of hemp polar lipidome. Analysis was performed by ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS), followed by bioinformatic lipid identification by Lipostar. To the best of our knowledge, a selective method suited to the suspect screening analysis of phospholipids and sulfolipids in *Cannabis sativa* was never reported so far.

## Materials and methods

### Chemicals

Ultra-pure water and ultra-pure methanol (MeOH) of HPLC-MS grade were obtained by Thermo Fisher Scientific (Waltham, MA, USA) and Romil Pure Chemistry (Pozzuoli, NA, Italy), respectively. Ammonium acetate, formic acid, acetic acid, trifluoroacetic acid (TFA), hydrochloric acid, and tetramethylammonium chloride (TMACl) were purchased from Merck (St. Louis, MO, USA). HPLC-grade chloroform ( $\text{CHCl}_3$ ), MeOH, and water used for sample pre-treatment were supplied by VWR International (Milan, Italy). Dichloromethane (DCM) was provided by Carlo Erba Reagents (Milan, Italy). The analytical standards 1,2-dilauroyl-sn-glycero-3-phosphocholine (PC (12:0/12:0)), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (PG (14:0/14:0)), 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) (PA (14:0/14:0)), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE (18:1/18:1) ( $\Delta^9$ -Cis)), 1-stearoyl-2-heptadecanoyl-sn-glycero-3-phospho-L-serine (sodium salt) (PS (18:1/18:1)), 1-(10Z-heptadecenoyl)-2-hydroxy-sn-glycero-3-phosphocholine (LPC (0/0:17:1)), 1-myristoyl-2-hydroxy-sn-glycero-3-phospho-1(1'-rac-glycerol) (sodium salt) (LPG (0:0/14:0)), 1-(10Z-heptadecenoyl)-2-

hydroxy-sn-glycero-3-(phospho-L-serine) (sodium salt) (LPS (0:0/17:1)), 1-myristoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (LPE (0:0/14:0)), N-myristoyl-D-erythro-sphingosylphosphorylcholine (SM (d18:1/14:0)), 3-O-sulfo-D-galactosyl- $\beta$ 1-1'-N-lauroyl-D-erythro-sphingosine (ammonium salt) (synthetic), (C12 mono-sulfo-galactosyl( $\beta$ ) ceramide (d18:1/12:0)), and SQDG were purchased from Avanti Polar Lipids (AL, USA). Stock standard solutions of single lipids were prepared in  $\text{CHCl}_3$  at a concentration of  $1 \text{ mg L}^{-1}$  and kept at  $-80^\circ\text{C}$ . The working standard solution containing all the considered lipids was prepared by diluting stock solutions in  $\text{MeOH/CHCl}_3$  (40/60, v/v) and used to evaluate recovery and matrix effect for the SPE procedure (concentrations of the single compounds are reported in Electronic Supplementary Material (ESM) Table S1).

## Instrumentation

All analyses were performed by ultra-high-performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry (HRMS). The LC system was a Vanquish chromatograph (Thermo Fisher Scientific) equipped with a binary pump, a thermostated column compartment and an autosampler (kept at  $14^\circ\text{C}$ ). A C18 Kinetex EVO column ( $100 \times 2.1 \text{ mm}$ , particle size  $1.7 \mu\text{m}$ , Phenomenex, Torrance, CA, USA) was employed for chromatographic separation by applying a method optimized for lipid analysis with slight modifications [23]. The injection volume was  $5 \mu\text{L}$ ; phase A and phase B were water and  $\text{MeOH/isopropanol}$  (80:20, v/v), respectively, both containing  $5 \text{ mmol L}^{-1}$  ammonium acetate and 0.02% (v/v) acetic acid; the total analysis time was 45 min and the following gradient was adopted: starting from 60%, B was first increased to 70% in 5 min and then to 99% in 25 min; the column was then washed at 99% B for 5 min and equilibrated at 60% B for 10 min. The flow rate and temperature were set at  $0.4 \text{ mL min}^{-1}$  and  $40^\circ\text{C}$ , respectively. Sample analyses were performed in triplicate and two blanks (neat solution of  $\text{MeOH/H}_2\text{O}$ , 60:40 v/v) were injected before each analyses batch to allow column conditioning and subtract blank contribution in data analysis.

The UHPLC system was coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The heated electrospray ionization (HESI) source was set as follows: capillary temperature  $320^\circ\text{C}$ ; sheath gas 35 arbitrary units (au); auxiliary gas 15 (au); spray voltage 2.5 kV; auxiliary gas heater temperature  $400^\circ\text{C}$ ; S-lens RF level 100%. The Q-Exactive mass spectrometer was operated in top 5 data-dependent mode. For full-scan spectra acquisition, the resolving power was set at 140,000 (full width at half-maximum, FWHM), scan range 200–1200  $m/z$ , automatic gain control target at  $5 \times 10^5$ , maximum ion injection time at 200 ms, and isolation window width of 2  $m/z$ . Tandem MS spectra were obtained by higher-energy collisional dissociation

(HCD) at a fixed collision energy of 40 normalized collision energy, with a resolution of 70,000 (FWHM), automatic gain control target of  $5 \times 10^5$  and dynamic exclusion of 6 s. External mass calibration of the spectrometer was carried out every 2 days, within a mass accuracy of 1 ppm, using the commercial Pierce positive and negative calibration solutions (Thermo Fisher Scientific). Spectra were acquired in positive and negative mode. Three technical replicates were performed for each sample. Raw data files were acquired by the Xcalibur software (version 3.1, Thermo Fisher Scientific).

## Lipid extraction from hemp

Inflorescences of seven individual *Cannabis sativa* plants of *Kompolti* variety were pooled, minced, and freeze-dried. The sample was divided into 1 g aliquots and stored at  $4^\circ\text{C}$  until extraction. The final lipid extraction procedure was based on a previous work, with some modifications [22]. One gram of hemp pooled sample was extracted three times using 20 mL of  $\text{MeOH/CHCl}_3$  (1:1 v/v), under vortex agitation for 40 min; the sample was centrifuged for 15 min at  $3000\times g$ ; the resulting three supernatants were pooled and evaporated to dryness by a IKA RV 8 rotary evaporator (IKA-Werke GmbH & Co. KG, Staufen, Germany). The residue was dissolved in 50 mL of  $\text{H}_2\text{O/MeOH}$  (80:20 v/v) with  $20 \text{ mmol L}^{-1}$  TFA and subjected to GCB-SPE. Three experimental replicates were performed and average chromatographic peak areas were used for relative quantitation.

## Polar lipid enrichment and fractionation by GCB-solid phase extraction

GCB cartridges were prepared by packing 250 mg of Supelclean ENVI-carb bulk sorbent (Sigma-Aldrich, St. Louis, MO, USA) between two polypropylene frits. The cartridge was conditioned by 5 mL of  $20 \text{ mmol L}^{-1}$  TFA in  $\text{DCM/MeOH}$  (80:20, v/v), 5 mL of  $20 \text{ mmol L}^{-1}$  TFA in  $\text{MeOH}$ , 10 mL of  $100 \text{ mmol L}^{-1}$  HCl in  $\text{H}_2\text{O}$ ; after equilibration with 10 mL of  $20 \text{ mmol L}^{-1}$  TFA in  $\text{H}_2\text{O/MeOH}$  (80:20, v/v), the hemp extract was loaded, and the cartridge was washed with 5 mL of  $20 \text{ mmol L}^{-1}$  TFA in  $\text{H}_2\text{O/MeOH}$  (80:20, v/v) and 500  $\mu\text{L}$  of  $\text{MeOH}$  (cartridge dead volume, used to remove residual water). Finally, two sequential elutions were performed, the first one using 15 mL of  $20 \text{ mmol L}^{-1}$  TFA in  $\text{DCM/MeOH}$  (80:20, v/v), the second one using 15 mL of  $20 \text{ mmol L}^{-1}$  TMAcI in  $\text{DCM/MeOH}$  (80:20, v/v) in back-flushing elution mode. Back-flushing elution was performed by inserting a Teflon piston with a Luer tip inside the cartridge, which was turned upside down and equipped with an empty tube on top of it for eluent addition. Before UHPLC-HRMS analysis, the eluates were dried at ambient temperature by means of a Centrivap concentrator (Labconco, Kansas City, MO, USA), reconstituted in 250

$\mu\text{L}$  of MeOH/CHCl<sub>3</sub>/isopropanol/H<sub>2</sub>O (47:3:30:20, v/v/v) and filtered through a GHP Acrodisc 0.2  $\mu\text{m}$  filter (PALL, Puerto Rico).

## Lipid identification

Lipostar (Molecular Discovery, Borehamwood, UK) was used for the identification of lipid species. It was employed to preprocess the raw data files, according to the settings reported in ESM Table S2, for baseline and noise reduction, peak extraction, smoothing, signal-to-noise ratio filtering, deisotoping, and deconvolution. Afterwards, lipids were identified by a database search. A customized database was built using the Lipid Builder Tool in the Lipostar DB Manager utility, by combining lipid head groups (phosphocholine, phosphatidic acid, phosphoethanolamine, phosphoglycerol, phosphoserine, sphingomyelin glycosylglycerol, and sulfoquinovosyl head groups) with the glycerol backbone and either 1 (lyso-forms) or 2 acyl chains, with length between C3–C35 and a number of 0–9 double bonds. Sphingomyelins (SM) were also included by combining the phosphocholine, phosphoethanolamine, and phosphoinositol heads with a ceramide group and an acyl chain. The database, which included exact masses and chemical formulae of lipid species, was complemented with theoretical MS/MS spectra by application of class- or compound-specific fragmentation rules, with product ions ranked as mandatory (compound-specific ions) or recommended (class-specific ions). The product ion classification in these two categories was built by combining literature data and experimental observations. Table 1 summarizes the mandatory and recommended product ions for all lipid classes, indicating their relative  $m/z$ , the molecular fragments associated, the ranking, and the references from which they were derived.

Lipids were identified by database search using a 5-ppm and 10-ppm tolerance for precursor ion masses and product ion masses, respectively.

For confidence discrimination of the identified lipids, the Lipostar classification system was initially considered to screen lipids identified with the highest scores. In this system, four stars are assigned to lipids identified with mandatory product ions, recommended product ions, and at least one fragment of a fatty acid chain; three stars are assigned to lipids identified with at least one mandatory product ion; two stars are assigned to lipids identified with at least one recommended product ion and one star if neither mandatory nor recommended product ions are identified, and the assignment is only based on the precursor exact mass. Only three- and four-star lipid identifications were maintained and manually checked to eliminate false positives. Finally, each identified lipid was ranked, based on the confidence levels previously reported [24–26], into level 1 (compounds identified by confirming both retention time and MS/MS spectra of a reference standard compound), level 2a (compounds putatively identified

only by MS analysis without standard reference compounds, with assigned *sn*-1 and/or *sn*-2 fatty acyl chain position), level 2b (compounds putatively identified only by MS analysis without standard reference and without discerning the position of the acyl chains), or level 3 (compounds assigned by a fragmentation pattern typical of the lipid class, but without information on the specific acyl chains, which is reported as sum composition). SQMG and lysophospholipids, whose specific acyl chain could be inferred from the molecular formula, as well as SQDG and phospholipids with two identical fatty acids, were assigned to level 2b instead of level 3. Moreover, level 2a was assigned to phospholipids with different peak intensities of the acyl chain fragment, as the most intense signal derives from the fatty acid in *sn*-2 position [27, 28].

## Statistical analysis

Each tested procedure was performed in triplicate. Analysis of variance (ANOVA) and Student's *t* test were employed to compare the different methods, in terms of the chromatographic areas of common detected lipids, in order to identify statistically significant differences. Excel 2016 was used to implement the ANOVA and *t* test functions and significance level was set at *p* value equal to 0.05.

## Semi-quantitative analysis

The average chromatographic peak areas, derived from triplicate analysis of the two GCB eluates, were integrated using Lipostar and normalized by a response factor, which took into account the instrumental response for the different polar lipid subclasses. In detail, the response factor was the ratio between the area of single reference standards (one standard for each lipid subclass), at a fixed concentration, and the area with the maximum intensity among all compounds; by this calculation, a number in the range 0–1 was assigned to each lipid class and used to normalize the peak areas. The obtained data were used to calculate percentages of the single compounds over the total normalized area.

## Results and discussion

### Development of the extraction procedure of polar lipids in hemp

Lipid extraction and fractionation were optimized starting from a procedure described earlier [22], which was suitably modified to maximize the total number of identified sulfolipids and phospholipids in hemp. The initial procedure involved an overnight extraction in isopropanol/MeOH (1:1, v/v), a SLE with MeOH/CHCl<sub>3</sub> and a final partitioning of the

**Table 1** Summary of the rules for lipid identification: product ions ( $m/z$ ), type of fragment, ranking, and references are indicated for each lipid species. The mass of the  $[sn-1-H]^-$  and the  $[sn-2-H]^-$  fragments depend on the fatty acid substituent and were not specified

| Lipid species | Product ion ( $m/z$ ) | Fragment                    | Ranking     | References      |
|---------------|-----------------------|-----------------------------|-------------|-----------------|
| PC/LPC        | –                     | $[sn-1-H]^-$                | Mandatory   | [28, 36–38]     |
|               | –                     | $[sn-2-H]^-$                |             |                 |
|               | 78.9590               | $[PO_3]^-$                  | Recommended |                 |
|               | 96.9696               | $[H_2PO_4]^-$               |             |                 |
|               | 168.04312             | $[C_4H_{11}NO_4P]^-$        |             |                 |
| PG/LPG        | –                     | $[sn-1-H]^-$                | Mandatory   | [23, 28, 36–39] |
|               | –                     | $[sn-2-H]^-$                |             |                 |
|               | 152.9958              | $[C_3H_6O_5P]^-$            |             |                 |
|               | 78.9590               | $[PO_3]^-$                  | Recommended |                 |
|               | 96.9696               | $[H_2PO_4]^-$               |             |                 |
|               | 227.0326              | $[C_6H_{12}O_7P]^-$         |             |                 |
|               | 209.0225              | $[C_6H_{10}O_6P]^-$         |             |                 |
| PA/LPA        | 171.0064              | $[C_3H_8O_6P]^-$            |             | [28, 36–38]     |
|               | –                     | $[sn-1-H]^-$                | Mandatory   |                 |
|               | –                     | $[sn-2-H]^-$                |             |                 |
|               | 152.9958              | $[C_3H_6O_5P]^-$            |             |                 |
|               | 78.9590               | $[PO_3]^-$                  | Recommended |                 |
| PE/LPE        | 96.9696               | $[H_2PO_4]^-$               |             | [27, 28, 36–38] |
|               | 171.0064              | $[C_3H_8O_6P]^-$            |             |                 |
|               | –                     | $[sn-1-H]^-$                | Mandatory   |                 |
|               | –                     | $[sn-2-H]^-$                |             |                 |
|               | 78.9590               | $[PO_3]^-$                  | Recommended |                 |
| PI/LPI        | 96.9696               | $[H_2PO_4]^-$               |             | [28, 36–38]     |
|               | 140.0118              | $[C_2H_7NO_4P]^-$           |             |                 |
|               | 196.0380              | $[C_5H_{11}NO_5P]^-$        |             |                 |
|               | –                     | $[sn-1-H]^-$                | Mandatory   |                 |
|               | –                     | $[sn-2-H]^-$                |             |                 |
|               | 241.0118              | $[C_6H_{10}O_8P]^-$         |             |                 |
|               | 78.9590               | $[PO_3]^-$                  | Recommended |                 |
|               | 96.9696               | $[H_2PO_4]^-$               |             |                 |
|               | 315.0487              | $[C_9H_{16}O_{10}P]^-$      |             |                 |
|               | 297.03809             | $[C_9H_{14}O_9P]^-$         |             |                 |
|               | 279.0275              | $[C_9H_{12}O_8P]^-$         |             |                 |
| PS/LPS        | 259.0225              | $[C_6H_{12}O_9P]^-$         |             | [28, 36–38]     |
|               | 223.0013              | $[C_6H_8O_7P]^-$            |             |                 |
|               | 297.03809             | $[M-H-R_1CO_2H-R_2CO_2H]^-$ |             |                 |
|               | –                     | $[sn-1-H]^-$                | Mandatory   |                 |
|               | –                     | $[sn-2-H]^-$                |             |                 |
| SM            | 152.9958              | $[C_3H_6O_5P]^-$            |             | [36]            |
|               | 78.9590               | $[PO_3]^-$                  | Recommended |                 |
|               | 96.9696               | $[H_2PO_4]^-$               |             |                 |
| SQDG/SQMG     | 78.9590               | $[PO_3]^-$                  | Mandatory   | [20, 23, 34]    |
|               | 168.0431              | $[C_4H_{11}NO_4P]^-$        |             |                 |
|               | –                     | $[M-CH_3-sn2 + H_2O]$       | Recommended |                 |
|               | 225.0069              | $[C_6H_9O_7S]^-$            | Mandatory   |                 |
|               | 80.9646               | $[HSO_3]^-$                 | Recommended |                 |
|               | 206.9969              | $[C_6H_7O_6S]^-$            |             |                 |
|               | 164.9863              | $[C_4H_5O_5S]^-$            |             |                 |
| 299.0436      | $[C_9H_{15}O_9S]^-$   |                             |             |                 |
| 243.0145      | $[C_6H_{11}O_8S]^-$   |                             |             |                 |

dry residue in H<sub>2</sub>O/MeOH/CHCl<sub>3</sub> (3:2:1, v/v/v), to separate polar and non-polar lipids. Each of the described stages was evaluated by comparing the number of identified lipids, as well as the chromatographic peak areas, indicating extraction recovery. The overnight extraction with isopropanol/MeOH (1:1, v/v) of the solid sample at 48 °C was described in previous works as useful to enhance the lipid diffusion into the extraction solvent [29, 30]. Nevertheless, the comparison of the extraction procedure with or without this step indicated a non-significant difference in the amount of extracted lipids. Moreover, the total number of identifications for the method without the overnight extraction was 92, versus only 72 lipids in the other case; lipid degradation due to the relatively high temperature applied in the overnight process could possibly explain the lower number of identifications in the latter experiments [31].

Then, the liquid-liquid repartition with H<sub>2</sub>O/MeOH/CHCl<sub>3</sub> (3:2:1, v/v/v) was evaluated; this procedure is commonly employed to fractionate polar lipids from non-polar lipids, due to their partitioning in the hydroalcoholic and chloroform phase, respectively. The necessity of this fractionation was verified to assess if any advantage could be achieved by removing non-polar lipids, for instance by reducing a possible ion suppression effect during UHPLC-MS/MS analysis; on the other hand, this additional sample manipulation could lead to loss of phospho- and sulfolipids. Therefore, the first MeOH/CHCl<sub>3</sub> (1:1 v/v) extract (reduced to 1 mL) and the two phases deriving from liquid-liquid repartition (each reduced to 500 µL) were analyzed and compared. In the MeOH/CHCl<sub>3</sub> extract, 100 polar lipids were identified, versus 22 and 70 lipid species detected in the hydroalcoholic and chloroform phases, respectively. Even though the total number of identified polar lipids was not significantly different, the procedure without liquid-liquid partitioning provided significantly higher chromatographic peak areas. This outcome indicated that, in the specific case of hemp, the separation of the polar lipid fraction from the non-polar lipid fraction is not only unnecessary but also detrimental for phospho- and sulfolipid analysis, due to depletion of lipid sample. In the light of these results, in the final procedure, lyophilized hemp was simply extracted with MeOH/CHCl<sub>3</sub> (1:1, v/v), and both the overnight SLE and liquid-liquid fractionation of polar lipids were removed from the final protocol. The result of this latter procedure (100 lipid identifications) was considered for comparison to the GCB enrichment and fractionation. Compared to the literature protocol, the procedure is not only faster but also easier to couple with the subsequent GCB enrichment, as the solvent could be easily evaporated at ambient temperature, avoiding depletion of lipid species [32].

### Phospholipid and sulfolipid fractionation on GCB

The GCB procedure was initially developed by recovery studies from spiked pure solvents, by applying a procedure used

for sulfolipid enrichment [22] and integrated with a further step for the analysis of phospholipids. In particular, two separate elutions were devised and tested on a standard mix (details in the “Materials and methods” section) to evaluate the efficiency of the fractionation process of phospholipids and sulfolipids. A mixture of 20 lipid standards containing 5 phospholipids, 8 lysophospholipids, 1 SM, 1 ceramide sulfate, and 5 SQDG was spiked into the loading solvent and the recovery was calculated (Table 2). Moreover, to assess the actual recovery (*R*) from the hemp extract, a real sample spiked at known concentration was subjected to the GCB-SPE and compared with a theoretical 100% recovery sample. More in detail, percentage recovery was calculated by the following equation:

$$R (\%) = 100 \times \frac{A_B - A_{NS}}{A_A - A_{NS}}$$

where *A*<sub>NS</sub>, *A*<sub>B</sub>, and *A*<sub>A</sub> are the peak areas obtained by analyzing a non-spiked sample (NS), a sample spiked before extraction (B), and a sample spiked after extraction (A), respectively. Percentage matrix effect (ME) was estimated as well, by the following calculation:

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

where *A*<sub>NS</sub>, *A*<sub>A</sub>, and *A*<sub>P</sub> are the peak areas obtained by analyzing extracts NS, A, and a neat standard solution in solvent (P), respectively. Satisfactory results were achieved, with recovery of the representative standards in the range 60–100% for most compounds and acceptable matrix effects for all the analytes, as shown in Table 2.

After studying the GCB-SPE procedure by recovery of spiked standards, the developed protocol was appended to the lipid extraction optimized previously. Initially, the GCB clean-up and enrichment were performed as previously described [21]: part of the extract (1.25 mL) derived from the treatment of 1 g of hemp was 1:20 diluted with 20 mmol L<sup>-1</sup> TFA in H<sub>2</sub>O/MeOH (80:20, v/v) and loaded, but results were significantly lower than the direct analysis (100 identifications), with detection of only 15 lipids in the first eluate, 14 of which were phospholipids, and 7 sulfolipids in the second eluate. Therefore, the whole extract was then used for the enrichment, after evaporation to avoid too large loading volumes. The results were significantly improved, with 79 lipids in the first eluate and 110 lipids in the second eluate. The separate analysis of the eluates indicated that the fractionation strategy developed using the GCB sorbent was successful, as 51 phospholipids were identified in the first fraction (65%) and 80 sulfolipids in the second fraction (73%). Compared to the direct analysis without enrichment on GCB, the total

**Table 2** Recovery of phospholipid and sulfolipid standards from pure solvents and spiked hemp extracts for the GCB-SPE procedure; matrix effect of the standards spiked on the hemp sample at known concentration

| Standard compound  | Recovery from pure solvent ( $\pm$ RSD, %) | Recovery from spiked hemp extract ( $\pm$ RSD, %) | Matrix effect ( $\pm$ RSD, %) |
|--|--|---|-------------------------------|
| First elution  |  |   |                               |
| PC (12:0/12:0)   | 95 $\pm$ 8                                 | 57 $\pm$ 2  | 106 $\pm$ 4                   |
| PG (14:0/14:0)   | 80 $\pm$ 4                                 | 45 $\pm$ 2  | 80 $\pm$ 7                    |
| PA (14:0/14:0)   | 105 $\pm$ 15                               | 64 $\pm$ 2  | 12 $\pm$ 1                    |
| PS (18:1/18:1)   | 113 $\pm$ 3                                | 51 $\pm$ 7  | 119 $\pm$ 10                  |
| PE (18:1/18:1)   | 71 $\pm$ 2                                 | 49 $\pm$ 5  | 73 $\pm$ 2                    |
| LPC (17:1/0:0)   | 80 $\pm$ 3                                 | 69 $\pm$ 1  | 111 $\pm$ 2                   |
| LPC (0:0/17:1)   | 102 $\pm$ 2                                | 84 $\pm$ 3  | 70 $\pm$ 5                    |
| LPG (14:0/0:0)   | 82 $\pm$ 0                                 | 95 $\pm$ 11                                       | 150 $\pm$ 5                   |
| LPG (0:0/14:0)   | 102 $\pm$ 12                               | 60 $\pm$ 1  | 165 $\pm$ 11                  |
| LPS (17:1/0:0)   | 100 $\pm$ 3                                | 80 $\pm$ 18                                       | 130 $\pm$ 10                  |
| LPS (0:0/17:1)   | 79 $\pm$ 5                                 | 80 $\pm$ 7  | 86 $\pm$ 3                    |
| LPE (14:0/0:0)   | 77 $\pm$ 2                                 | 100 $\pm$ 8                                       | 68 $\pm$ 9                    |
| LPE (0:0/14:0)   | 103 $\pm$ 8                                | 84 $\pm$ 6  | 98 $\pm$ 4                    |
| SM (d18:1/14:0)  | 84 $\pm$ 1                                 | 62 $\pm$ 4  | 102 $\pm$ 6                   |
| Second elution   |  |   |                               |
| C12 mono-sulfo-galactosyl( $\beta$ ) ceramide (d12:0/18:2) | 75% $\pm$ 4                                | 79% $\pm$ 4                                       | 95% $\pm$ 10                  |
| SQDG (18:3/18:3)   | 60 $\pm$ 1                                 | 69 $\pm$ 1  | 55 $\pm$ 1                    |
| SQDG (16:0/18:2)   | 63 $\pm$ 2                                 | 76 $\pm$ 3  | 59 $\pm$ 3                    |
| SQDG (16:0/16:0)   | 50 $\pm$ 4                                 | 52 $\pm$ 2  | 51 $\pm$ 3                    |
| SQDG (16:0/18:3)   | 66 $\pm$ 0                                 | 71 $\pm$ 5  | 73 $\pm$ 3                    |
| SQDG (14:0/18:3)   | 66 $\pm$ 1                                 | 78 $\pm$ 5  | 70 $\pm$ 4                    |

number of polar lipid species was definitely increased; in addition, the chromatographic peak areas of the detected species were significantly enhanced, demonstrating an efficient clean-up from interferent species, which could suppress ionization during HESI.

Finally, the two GCB eluates were analyzed after pooling, to assess if the number of UHPLC-MS/MS runs could be reduced without loss in qualitative identification of the target lipid species. From these experiments, 106 lipids were identified, a significantly lower number compared to the analysis of the two separate fractions and consistent with results from the direct analysis without enrichment. Along with the smaller number of identified lipids, the peak areas in the pooled sample resulted to be significantly lower than the areas in the two separate eluates. This result indicated that ion suppression could occur among sulfolipids and phospholipids, depending on their concentration, thus showing the advantages of performing two different elutions and analyses. If this result could be theoretically expected, as fractionation reduces sample complexity, thus improving detection and identification, it actually indicated that the fractionation of lipids into individual lipid classes could be a valuable strategy for deeper mining of lipids in complex matrices.

## Lipid identification in hemp extracts

The bioinformatic identification of lipid species was accomplished using Lipostar [33], as it allows untargeted high-confidence identification of lipids in complex samples. In the case of hemp, a database for phospholipid and sulfolipid species was compiled for the identification of these target lipid classes. For sulfolipid identification, the specific classes of SQDG and SQMG were included in the custom database [23]. Diagnostic ions for MS/MS spectra matching were selected based on previous works [20, 34] and on relative intensity in the experimental conditions; these ions were ranked into mandatory and recommended ions based on whether they were compound or class specific. Thanks to the application of the fragmentation rules, summarized in Table 1, phospholipids, sulfolipids, and the other polar lipid classes were identified. The most abundant lipids detected in the hemp sample are reported in Table 3, while comprehensive tables, indicating the whole set of lipids identified in all tested procedures, are reported in the ESM (Tables S3, S4, S5, and S6 enclose the total identified species in the solid-liquid hemp extract, in the first eluate of GCB-SPE, in the second eluate of the GCB-SPE and in the pooled eluate, respectively).

It is worth noticing that the use of RP chromatography allowed to resolve isomers and improved the total number of

**Table 3** Identification of the most abundant phospholipids and sulfolipids detected in the first and second GCB eluate, respectively

| Measured mass | Molecular formula                                 | Adduct  | Fragment ions common to phospholipids | Fragment ions common to one class of phospholipids         | Fragment ions common to sulfolipids             | Fragment ions common to one class of sulfolipids | Fragment ions depending on fatty acid composition | Identity (confidence level) |
|---------------|---|---|---------------------------------------|--|---|--|---|-----------------------------|
| First eluate  |   |   |                                       |  |   |  |   |                             |
| 780.5534      | C <sub>44</sub> H <sub>78</sub> NO <sub>8</sub> P | [M + H] <sup>+</sup> [M + CH <sub>3</sub> COO] <sup>-</sup>                       | 78.9586; 152.9957                     | 104.1068; 124.9996; 168.0430; 184.0730; 224.0693           | 80.9647; 164.9861; 206.9967; 225.0073; 299.0442 | 277.2173; 279.2330                               | 277.2173; 279.2330                                | PC(18:3/18:2) [2a]          |
| 782.5691      | C <sub>44</sub> H <sub>80</sub> NO <sub>8</sub> P | [M + H] <sup>+</sup> [M + Na] <sup>+</sup> [M + CH <sub>3</sub> COO] <sup>-</sup> | 78.9586                               | 104.1068; 124.9996; 146.9818; 168.0430; 184.0730; 224.0693 | 80.9647; 164.9861; 206.9967; 225.0073; 299.0442 | 279.2330; 465.2379                               | 279.2330; 465.2379                                | PC(18:2/18:2) [2a]          |
| 756.5536      | C <sub>42</sub> H <sub>78</sub> NO <sub>8</sub> P | [M + H] <sup>+</sup>  |                                       | 104.1068; 124.9996; 184.0730                               |   |  |   | PC(34:3) <sup>a</sup> [3]   |
| 483.2729      | C <sub>22</sub> H <sub>45</sub> O <sub>9</sub> P  | [M-H] <sup>-</sup>  | 78.9586; 152.9957                     | 171.0063; 245.0433   |   | 255.2329   | 255.2329  | LPG(0:0/16:0) [2b]          |
| 481.2569      | C <sub>22</sub> H <sub>43</sub> O <sub>9</sub> P  | [M-H] <sup>-</sup>  | 78.9586; 96.9694; 152.9957            | 171.0064; 245.0430   |   | 253.2173   | 253.2173  | LPG(0:0/16:1) [2b]          |
| Second eluate |   |   |                                       |  |   |  |   |                             |
| 555.2843      | C <sub>25</sub> H <sub>48</sub> O <sub>11</sub> S | [M-H] <sup>-</sup>  |                                       |  | 80.9647; 164.9861; 206.9967; 225.0073; 299.0442 | 255.2332   | 255.2332  | SQMG(0:0/16:0) [2b]         |
| 577.2689      | C <sub>27</sub> H <sub>46</sub> O <sub>11</sub> S | [M-H] <sup>-</sup>  |                                       |  | 80.9647; 164.9861; 206.9967; 225.0073; 299.0442 | 277.2174   | 277.2174  | SQMG(0:0/18:3) [2a]         |
| 813.4831      | C <sub>43</sub> H <sub>74</sub> O <sub>12</sub> S | [M-H] <sup>-</sup>  |                                       |  | 80.9647; 164.9861; 206.9967; 225.0073           | 255.2331; 275.2019; 537.2739; 557.2426           | 255.2331; 275.2019; 537.2739; 557.2426            | SQDG(16:0/18:4) [2b]        |
| 815.4983      | C <sub>43</sub> H <sub>76</sub> O <sub>12</sub> S | [M-H] <sup>-</sup>  |                                       |  | 80.9647; 164.9861; 206.9967; 225.0073           | 255.2332; 277.2174; 537.2740; 559.2583           | 255.2332; 277.2174; 537.2740; 559.2583            | SQDG(18:3/16:0) [2b]        |
| 793.4415      | C <sub>37</sub> H <sub>66</sub> O <sub>12</sub> S | [M + CH <sub>3</sub> COO] <sup>-</sup>  |                                       |  | 80.9647; 164.9861; 206.9967; 225.0073           | 283.2645; 565.3052                               | 283.2645; 565.3052                                | SQDG(10:2/18:0) [2b]        |

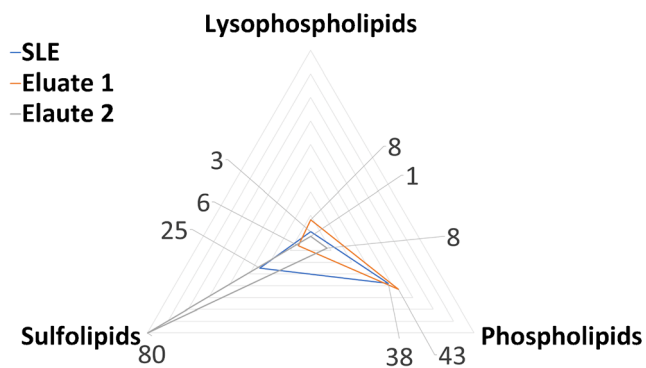
<sup>a</sup> Only the sum composition was indicated when no diagnostic fragments were detected for the acyl chains



identifications. In fact, positional regioisomers, with respect to the fatty acid acyl chains, possess the same exact mass and fragmentation pattern but can be distinguished by the relative abundance of the fragments, arising from the loss of the fatty acid chains. For example, the PG (16:1/18:3) fragmentation pattern allowed to define the appropriate fatty acid position in the glycerol backbone (ESM Fig. S1); the higher abundance of the ion at  $m/z$  277.2173, compared to the ion at  $m/z$  253.2175 corresponding to the loss of the fatty acid at *sn*-2 and *sn*-1 position respectively, led to distinguish PG (16:1/18:3) from its isomer PG (18:3/16:1) [28]. Concerning lipid lyso-forms, and in particular the SQMG subclass, the ion at  $m/z$  243.0181 enabled to assess the position of the fatty acid in the glycerol backbone. As reported in the literature [20], the presence of this ion in the MS/MS spectra is diagnostic for SQMG presenting the fatty acid at the *sn*-1 position (ESM Fig. S2 displays the chromatographic resolution of the two isomers). The radar chart in Fig. 1 displays the distribution of the identified lipid species in the simple SLE extract and in the two eluates deriving from the GCB-SPE. Mostly phospholipids (51 identifications) and sulfolipids (80 identifications) were identified in the first and second eluates, respectively; instead, by applying the simple SLE, both lipid classes were determined. Further insight into the distribution of lipid species was provided by the Venn diagram (Fig. 2), which indicated that most of the lipids identified by the simple SLE procedure (67 lipid, 73%) were in common with the two fractions; as far as lipids common to both GCB eluates were concerned, the juxtaposition was only for 19 lipids, which represented approximately the 10% of the identified lipids in both eluates. The result further supports the orthogonality achieved by the double elution GCB protocol.

### Semi-quantitative analysis

In order to provide an estimation of the most concentrated species detected in the hemp sample, a semi-

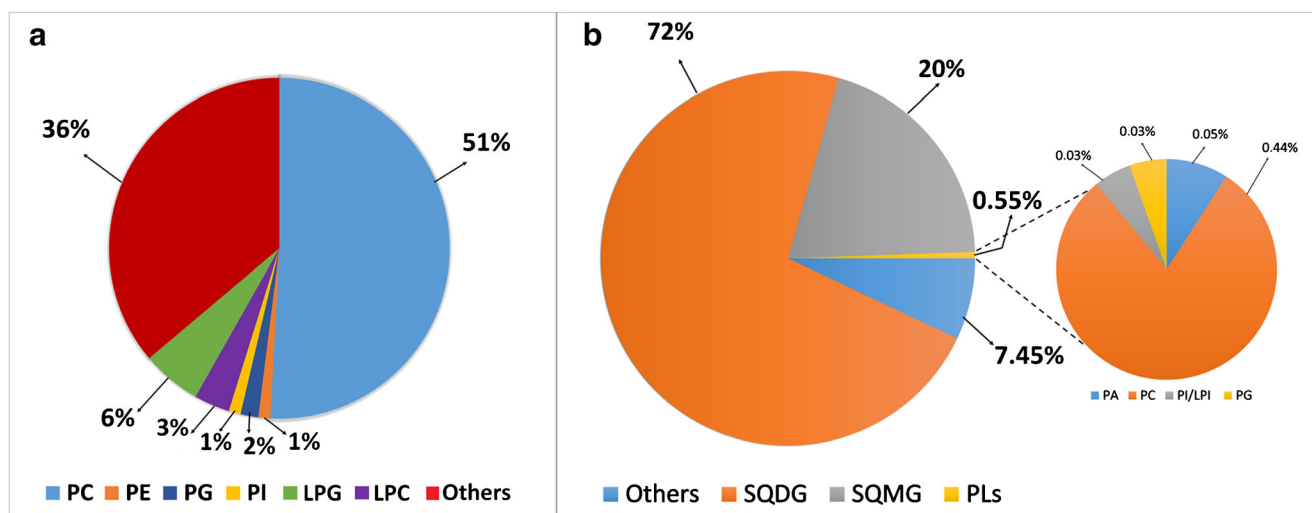


**Fig. 1** Radar chart displaying the distribution of the identified lipid species, divided into sulfo-, lyso-, and phospholipids, in the simple SLE extract (blue line), in the first eluate (orange line) and second eluate (gray line) of the GCB-SPE



**Fig. 2** Venn diagram indicating the distribution of the identified lipids for the direct SLE procedure and the two GCB-SPE eluates; the total number of lipids identified for each category is reported in brackets

quantitative analysis was performed. This analysis allowed to obtain the relative abundance of the identified compounds for each eluate; the relative abundance was calculated as the normalized individual peak area of each lipid species over the total area of all lipids and was expressed as a percentage (ESM Table S7). Relative lipid abundances in the hemp sample are reported in the pie charts (Fig. 3); PCs represented the predominant lipid class in the first eluate, accounting for the 51% of the hemp phospholipidome. It is worth noting that the fatty acyl chain combinations 16:0, 16:1, 18:2, and 18:3 were the most abundant (ESM Fig S3c). Indeed, PC (18:2/18:2), PC (18:3/18:2), PC (16:0/18:2), and PC (18:2/18:1) represented 13%, 10%, 5%, and 3% respectively [35]. The sulfolipid classes almost typified the whole second enriched fraction (pie chart B, Fig. 3), which consisted in 72% SQMGs and 20% SQDGs. In particular, 10% was composed of SQMG (0:0/16:0), 35% of SQDG (18:3/16:0), and 8% of SQDG (16:0/16:0) as reported in ESM Table S7. The fatty acyl chain combinations including the 16:0, 18:1, and 18:3 chains were the most abundant not only in the case of phospholipids but also for the SQMG and SQDG subclasses (ESM Fig S3a and S3b). The estimation of the lipidome composition allowed to notice the similarity with other plants, such as parsley [20] and spinach [34], as well as other related matrices, such as spirulina microalgae [22], which is a photosynthetic microorganism. In all these matrices, SQDG (16:0/18:3) and SQDG (16:0/16:0) were the predominant sulfolipids, similarly to the compounds identified in the hemp matrix.



**Fig. 3** Relative abundances (%) of the identified lipid species in the first GCB-SPE eluate (a) and second eluate (b) reported according to main lipid classes. PL, phospholipids

## Conclusions

The polar lipids composition of hemp was never investigated before, due to the extremely low concentration of lipid species in this plant. The present work dealt with the development of an extraction-enrichment procedure capable of fractionating and identifying a large number of polar lipids, especially phospholipids and sulfolipids, by exploiting GCB clean-up capabilities. Different sample extraction procedures were compared: the most efficient, in terms of both number and amount of identified lipids, involved the minimum sample handling before SPE. The adsorption mechanism on the GCB surface was successfully employed to selectively elute different species in two fractions, in order to maximize the number of identifications, due to the reduction of matrix effects and consequent lower suppression of the less concentrated species. The tentative identification of 189 polar lipids was achieved by LC-HRMS analysis combined with the Lipostar software and the relative abundances were also evaluated to estimate the most concentrated species, which resembled literature data on other vegetable matrices. The proposed methodology is simple and provides a reliable identification, allowing the first polar lipidome characterization of the hemp matrix.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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