A rapid LC–MS/MS method for quantitative profiling of fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grapes

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Abstract

The abundance of lipids in plants is influenced by genotype and phenotype. Despite being a very important class of plant metabolites, knowledge of grape lipids is still very limited to date, with the exception of those located in seeds. The few investigations of grape lipids have shown that their profile depends on grape maturity, the variety and their location in the berry. Recent advances in liquid chromatography coupled to mass spectrometry have paved the way for faster analysis of lipids with minimal sample preparation. Here we describe a validation method for the extraction, identification and quantification of different classes of grape lipids: fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids using liquid chromatographic electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). The method was validated for 33 lipids, with linearity range (R2=0.95-1.00), LOQ (0.003 – 14.88 $ngmL^{-1}$) and intraday and interday repeatability being evaluated for each lipid. The lipid profiling method developed was successfully applied to the analysis of 18 grape samples (10 red grape and 8 white grape varieties) from 4 different genetic groups: *Vitis vinifera*, *Vitis* non-*vinifera*, Muscat and hybrid; 32 lipids were identified and quantified. This method, which can be easily expanded to include further compounds and other plant tissues, is the starting point for analysis of the lipid profile in different grape tissues, an essential goal for better understanding the role of lipids in grape physiology.

Keywords Lipidomics; sterols; glycerophospholipids; glycerolipids; fatty acids; grape

1. Introduction

Lipids have an essential role in all plant cells, in terms of structure and organisation, signalling events, protein regulation, metabolic transformation and trafficking [1]. The vegetative cells of plants contain from 5% to 10% of lipids (dry weight), and almost all of this weight is found in the membranes [2]. Although each square centimetre of a plant leaf may contain only 0.2 mg of lipids, the lipid membranes are the main barriers delineating the cell and its compartments and they form the sites where many essential processes occur, including light-harvesting and the electron transport reactions of photosynthesis [2]. Epidermal cells produce cuticular lipids that coat the surface of plants, providing the crucial hydrophobic barrier that prevents water loss and also providing protection against pathogens and other environmental stresses [3]. Furthermore, lipids and lipid metabolites released from membranes work as signal molecules in activating the plant defence response [4]. However, the study of lipids has been complicated due to their structural diversity and complexity. Following the classification proposed by LIPID MAPS (http://www.lipidmaps.org), lipids can be classically divided in eight categories, namely fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PL), saccharolipids (SL) and polyketides (PK) [1]. Furthermore, each of these categories includes distinct classes and sub-classes, once again increasing the complexity of this family of compounds. The most abundant types of lipids in plant cells are those deriving from fatty acid and glycerolipid biosynthetic pathways; for example, small amounts of fatty acids are important as precursors for the hormone jasmonic acid and in the acylation of certain membrane proteins. Other classes of lipids derive from the isoprenoid pathway, such as sterols [2]. Glycerophospholipids are the main constituent in the cellular membrane and understanding lipid changes is useful for understanding cell function [5].

Little research has been conducted on grape lipids, with the exception of grape seed lipid composition, and therefore knowledge of grape lipid composition is still very limited and there are only a few studies on the topic. The relationship between the evolution of grape constituents during berry development and ripening was studied by Rubio et al [6]. Roufet et al. studied the effects of maturation on fatty acid content in grapes, Le Fur et al. studied the evolution of phytosterols in grape berry skins during the last stages of ripening, while Barron et al. found a relationship between triglycerides and grape ripening indices [7,8]. Studies related to the fatty acid composition of different tissues in grapes have shown that linolenic acid is predominant in leaves, while linoleic acid is most abundant in pericarps and phospholipids are present in larger quantities in the skin and pulp [9]. The composition of lipids has been analysed in different tissues, such as the leaves, pericarp, skin and seeds of Cabernet Sauvignon grapes, and the fatty acid profile of *Vitis labrusca* and *Vitis vinifera* grapes was analysed in different components [9,10].

The plasma membrane and microsomal fraction in grape leaves have been isolated because of their importance as an indicator of environmental changes, while sterol, phospholipid and sphingolipid composition have been discussed in relation to membrane fluidity in grapevine leaves [11]. Grape seed oil is rich in unsaturated fatty acids such as linoleic and oleic acid and thus offers many advantages for human consumption; the food, pharmaceutical and cosmetic industries have shown great interest in grape seed oil due to its exceptional antioxidant properties [12]. Because of its nutritional and therapeutic properties, grape seed oil has been analysed and the main components in terms of oil, fatty acids, vitamin E, active compounds and phytosterol composition quantified in several varieties [13,14]. Fatty acids, sterols and the triacylglycerol composition of grape seed oil has been evaluated in grapes from different countries (France, Italy and Spain), making it possible to distinguish the origin of the oil based on lipid profiling using GC-MS analysis [15]. Matrix-assisted-laser-desorption-ionization mass spectrometry (MALDI-MS) was used by De Marchi and colleagues [16] in order to characterise the triacylglycerol (TAG) composition of grape seed oils, in a very short run time, obtaining useful information about grape varieties and processing conditions.

Grape lipids are important factors in oenology since they are capable of modulating the yeast metabolism. This is of major importance in the case of white and rosé winemaking, where lipids can be a limiting factor due to the short contact with the grape skins. Grape lipids are essential during fermentation in order to limit the production of excessive amounts of acetic acid from acetic acid metabolism, favouring the penetration of amino acids into the yeast cell [17–19]. Ergosterol, other sterols and certain long-chain fatty acids such as oleanolic acid and oleic acid are known to act as "growth factors", increasing the yeast population and accelerating the fermentation speed under complete anaereobiosis. Moreover, they have been shown to act as "survival factors", since yeasts well supplemented in these grape lipids are more viable and are thus capable of prolonging their fermentation activity [19]. In other words, grape lipids can induce nutritional limitation of yeast activities, a complex factor which is more severe in musts with a concomitant deficiency in assimilable nitrogen [20]. In conclusion, our current understanding of yeast physiology shows the need for a metabolite profiling method capable of quantitatively screening for grape lipids [20,21].

Several analytical methods using numerous techniques have been developed to investigate lipids, including thin-layer chromatography, gas chromatography and HPLC-UV, but because of the complexity of this family of compounds, recently mass spectrometry has become the leading technology for rapid lipidomic analysis [22], due to its good sensitivity, specificity and dynamic range. Recent developments achieved in liquid chromatography, such as ultra high performance liquid chromatography (UHPLC) and the availability of new versatile stationary phases, has made this technique suitable for lipid profiling. Thus UHPLC-MS based approaches can help to expand the number and classes of lipids that can be analysed by offering new standards in terms of sensitivity and selectivity[23]. Electrospray ionization

(ESI) is the most widely used ionization technique for the analysis of lipids: in positive mode, the observed MS spectra are dominated by protonated molecules, [M+H]+ or other ionic species, due to the high tendency of lipids to form adducts with sodium, potassium and ammonium ; in negative mode, the deprotonated molecule [M−H]− and some acetate and/or formate adducts are generally observed [24,25]. The use of high resolution shotgun lipidomics allows the identification of different triglycerides in seed oils [26]. Finally, an interesting approach for the study of subcellular lipidomics is MALDI imaging [27].

To our knowledge, reports on extensive method validation for lipid profiling analysis using LC-MS/MS in complex grape matrices are limited, and the data published to date are only available for grape seed oil composition [14,16]. Here we propose a sensitive and accurate LC-tandem-MS-based method for the simultaneous determination and quantification of multiple classes of lipids such as fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids in grape samples. Application of this method could greatly enhance knowledge of the role of grape lipids for different technological and biological questions regarding grape growing and winemaking.

2. Experimental method

2.1 Chemicals and materials

Standards were purchased from Sigma–Aldrich (Sigma–Aldrich, Milan, Italy) and Avanti Polar Lipids (Alabaster, AL). The chemicals Acetonitrile (ACN, LC–MS grade), 2-propanol (IPA), methanol (CH_3OH, LC–MS grade) and chloroform ($CHCl_3$) were purchased from Sigma–Aldrich. Formic acid (HCOOH) and ammonium formate (NH_4COOH) additive for LC–MS were also from FLUKA Sigma–Aldrich. All aqueous solutions, including the HPLC mobile phase, were prepared with water purified using a Milli-Q system (Millipore, Vimodrone, Milan, Italy).

The following chemical standards of lipids were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy): oleoyl-L-carnitine hydrochloride, palmitoyl-L-carnitine hydrochloride, desmosterol, ergosterol, lanosterol, uvaol, arachidic acid, behenic acid, *cis*-11-eicosenoic acid, erucic acid, heptadecanoic acid, margaric acid, lignoceric acid, linoleic acid, linolenic acid, myristic acid, myristoleic acid, oleanolic acid, oleic acid, *cis*-vaccenic acid, palmitic acid, palmitoleic acid, stearic acid, 1,2,3-tripentadecanoylglycerol 1-linoleoyl-rac-glycerol, 1-monopalmitoleoyl-rac-glycerol, 1-oleoyl-rac-glycerol, glyceryl trioleate, glyceryl tripalmitoleate, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-

glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt, 1-palmitoyl-sn-glycero-3-phosphocholine, ceramide. Docosahexaenoic acid and cholesterol bought from Aldrich-Fluka-Sigma S.r.l. (Milano, Italia), were chosen as internal standards (IS).

Butylated hydroxytoluene (BHT), provided by Aldrich-Fluka-Sigma S.r.l. (Milano, Italia), was used as an antioxidant during the extraction steps.

2.2. Preparation of lipid standards

All analytical standards used for method development are listed in Table S1 with the relative molecular formula and Chemspider ID.

Standard stock solutions of lipids were prepared individually at a concentration range from 1 to 10 mg mL^{-1} in a $CHCl_3/CH_3OH$ mixture (2:1 v/v) in dark glass tubes. A working standard solution of each analyte was prepared from the stock solution by appropriate dilution in ACN/IPA/H_2O mixture (65:30:5 v/v/v) and employed for development and validation of the method.

A combined standard spiking solution of all analytes was prepared by diluting the respective stock solutions in ACN/IPA/H_2O (65:30:5 v/v/v) and this was diluted again in order to obtain 21 points with decreasing concentrations of each analyte, in the range of concentrations reported in Table 1.

IS solutions of docosahexaenoic acid and cholesterol were prepared at concentrations of 100 µg mL^{-1} and 1 µg mL^{-1} respectively and used to check sample preparation efficiency.

The stock of standard solutions and the spiking solutions were stored at −20 °C.

2.3 Sample collection

Grapes samples were from the “Fondazione Edmund Mach” grape germplasm collection (ITA362), located in San Michele all'Adige, Italy (46°18’ N, 11°13’ E). All plants were grafted onto the rootstock Kober 5BB in five replicates and trained using the Guyot system. Samples of healthy grapes from 10 red and 8 white grape varieties (Table S2) were harvested at technological maturity in the same year (2010) and were analysed using the method developed.

2.4 Sample preparation

Grape powder was prepared as previous described by Gika et al [28] and kept at -80 °C. Lipids were extracted according to Folch's method [29]. A precise amount of 0.5 (±0.005) g from each sample was weighed and 1.5 mL of CH_3OH were added, vortexed for 30 s, 3 mL of $CHCl_3$ containing butylated hydroxytoluene (BHT 50 mgL^{-1}) were added, followed by the addition of 10 µL of internal standard (IS) (docosahexaenoic acid 100 µg mL^{-1}). Samples were placed in an orbital shaker for 60 min. Afterwards 1.25 mL of water was added. After 10 min the samples were centrifuged at 3600 rpm for 10 min. The total lower lipid-rich layer was collected. The second extraction was performed using 2 mL of $CHCl_3/CH_3OH/H_20$ 86:14:1 v/v. The samples were centrifuged at 3600 rpm for 10 min, the total lower lipid-rich layer was collected. Both fractions were unified and evaporated to dryness under N2. Samples were reconstituted in 300 µL of ACN/IPA/H_2O (65:30:5 v/v/v) containing the IS (cholesterol at a concentration of 1 µg mL^{-1}) before LC/MS/MS analysis. Five microliters of sample were injected into the LC/MS system. Each sample was diluted 1:100 with ACN/IPA/H_2O (65:30:5 v/v/v) and re-injected for quantification of the most abundant lipids.

2.5 Instrumental conditions

Separation was performed using a UHPLC Dionex 3000 (Thermo Fisher Scientific Germany), with a RP Ascentis Express (15 cm X 2.1 mm; 2.7 µm C18) purchased from Sigma (Milan, Italy). Column temperature was set at 55 °C using a Peltier effect column oven (Dionex Thermo Fisher Scientific Germany). Samples were injected using an autosampler (Dionex Thermo Fisher Scientific Germany) set at 10 °C. Separation was carried out following a 30 min multistep linear gradient as reported by Hu et al [30]. From 0-1.5 min isocratic elution with 32% B; from 1.5 to 4 min increase to 45% B, then to 52% B in 1 min, to 58% B in 3 min, to 66% B in 3 min, to 70% B in 3 min, to 75% B in 4 min, to 97% B in 3 min, then 97% B was maintained for 4 minutes. From 25.0 to 25.1 min solvent B was decreased to 32% and then maintained for another 4.9 min for column re-equilibration. Total duration of the analysis was 30 minutes, including post-time. The flow-rate was 0.26 $mLmin^{-1}$, mobile phase A consisted of ACN 40% in water, NH_4COOH 10 mM and HCOOH 0.1% and B consisted of IPA 90%, ACN 10%, NH_4COOH 10 mM and HCOOH 0.1%.

The HPLC system was coupled directly to an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a Electro Spray source. AnalystTM software version 1.6.1 (Applera Corporation, Norwalk, CT, USA) was used for instrument control and data acquisition. The transitions and spectrometric parameters were optimized individually for each standard by direct infusion of their solutions (10 µg mL^{-1}) in water/ACN (40:60 v/v) with NH_4COOH 10 mM and HCOOH 0.1% into a mass spectrometer at a flow rate of 10 µL min^{-1}. The two most abundant fragments to use as quantifier and qualifier were identified for each compound. Declustering potential (DP) and entrance potential (EP) were optimized for each precursor ion and collision energy (CE) and Collision Cell Exit Potential (CXP) for each product ion. Table 2 shows the compound-specific instrumental parameters used in the analytical method. The presence of our metabolite of interest was confirmed using the q/Q ratio [31]. The spray voltage was set at 5500 V for positive mode and -4500 V for negative mode. The source temperature was set at 250°C, the nebulizer gas (Gas 1) and heater gas (Gas 2) at 40 and 20 psi respectively (1 psi = 6894.76 Pa). UHP nitrogen (99.999%) was used as both curtain and collision gas (CAD) at 20 and 9 psi.

2.6 Method validation

The method validation assays were performed according to the currently accepted US Food and Drug Administration (FDA) bio-analytical method validation guide [32]. Validation assays were established on calibration standards and quality control (QC) samples prepared as a pool of grape samples and extracted according to the procedure described above. The standard mix was used for calibration curves and in order to establish the LOQ. The QC sample was used in order to evaluate the stability of samples during analysis, intra-day and inter-day variability and to evaluate the performance of the extraction method in the real samples.

2.6.1 Linearity, limit of detection, limit of quantification and matrix effect

Calibration curves were made in pure solvent (ACN/IPA/H_2O) and matrix extract (QC). To evaluate the percentage of matrix effect for each compound, calibration curves were compared. Matrix effect values were determined by comparing solvent and matrix-matched calibration curves in terms of slope ratios :%ME=100% x (1 – slope solvent/ slope matrix) as shown in Table 1 [33].

To assess linearity, three independent calibration curves were prepared by adding increasing concentrations of each lipid in different concentration ranges, as shown in Table 1. The limits of quantification (LOQ) and limits of detection (LOD) were evaluated at the concentration in which the quantifier transition presented a signal to noise (S/N) ratio of >10 and >3 respectively.

2.6.2 Intra-day and inter-day variability

To determine intra-day and inter-day variability, 10 replicates of a QC sample middle concentration level were extracted and injected on the same day and then re-injected for 5 consecutive days. Intra-day and inter-day variability were evaluated by calculating the coefficients of variation (CV %) as shown in Table 3. CV % should not exceed a value of 15% for intra-day assay and 20% for inter-day assay.

2.6.3 Recovery, precision and accuracy

The QC sample was used for method validation, by spiking it with known amounts of each lipid, corresponding to three different points (low, middle and high) in the calibration range for each analyte. The recovery test was estimated on ten spiked grape samples and calculated as the average of the "measured value/expected value" ratio (%) (Table 3).

Accuracy and precision were calculated by analysing ten QC spiked samples, corresponding to a medium point. Precision was reported as the Relative Standard Deviation (RSD) between the replicate measurements in spiked QC samples, while accuracy was reported as the Relative Error (RE), which was calculated as the difference between the measured value and the theoretical value, divided by the theoretical value and expressed as a percentage (Table 3).

2.6.4 Application of the method to grape samples

The applicability of the proposed analytical method was tested by measuring the levels of standard lipids in 18 samples of grapes (10 red grape and 8 white grape varieties) from 4 different genetic groups [34]: *Vitis vinifera*, *Vitis* non-

vinifera, Muscat and hybrid (Table S2). All samples were harvested at technological maturity and were analysed using the method developed.

3. Results and discussion

In contrast with the other main components of plants (proteins, carbohydrates and nucleic acids), lipids are defined on the basis of their physical properties, rather than their common chemical structure. Thus lipids are often loosely defined as compounds that are insoluble in water and that can be extracted from cells using non-polar organic solvents (such as chloroform). The most abundant types of lipid in most cells, however, are those deriving from the fatty acid and glycerolipid biosynthetic pathway, and these are the lipids we took into consideration in this analysis, together with sterols and glycerophospholipids [2].

3.1 Reverse phase liquid chromatography (LC)

Lipid analysis takes different approaches into consideration: liquid chromatography mass spectrometry or fast infusion analysis for example, although the use of liquid chromatography is more useful for the quantification of low-abundance lipids because separation can enrich low-abundance molecular species and exclude the interaction of many lipid species, while chromatographically separating isobaric compounds. With these methods column separation facilitates the identification of isomeric species with identical fragmentation patterns, whereas shotgun lipidomics in this field encounter some difficulties. Many reviews have reported the application of LC-MS to the quantification of low-abundance lipids [35]. In Fig. 1 we show that the different classes of grape lipids are well divided on the basis of retention time in the instrumental conditions described in section 2.5, while there is good separation of fatty acids on the basis of chain length and unsaturation. The sterols are well clustered in the central part of chromatogram, except for uvaol and oleanolic acid, which are C30 sterols with a different steric distribution and interaction with the column. Class separation using liquid chromatography (LC) followed by MS detection for species identification is very important in the analysis of glycerophospholipids extracted from a complex biological matrix [36–38]. Glycerophospholipids are distributed in the central part of the chromatogram, except for 1-palmitoyl-sn-glycero-3-phosphocholine, as the retention time indeed also depends on the alkyl chain. At the end of chromatogram we find

triacylglycerols in the glycerolipids category, which are less polar because of the long alkyl chain and the absence of a polar group; monoacylglycerols are instead found in the first 10 minutes of the chromatogram. Finally, we can observe that ceramide has a retention time of 19.2 min because of the d18/1/18/0 chain. A typical chromatogram of separation is shown in Fig 2.

3.2. Fragmentation study and optimization of MS/MS conditions

The classification of long-chain lipid substances into different families is useful because mass spectrometric methods developed for lipid analysis have been centred around these divisions [24].

In our method, structural information to confirm lipid identity was obtained by means of a preliminary fragmentation study, showing a characteristic fragmentation pattern for each class of compounds.

Q1 scan spectra of each lipid showed different ion species, due to the presence of ammonium formiate in the mobile phase buffer and the possible consequent formation of adduct ions.

Fatty acids (FA) were typically analysed as negative ions using ESI. For almost all of them, the adduct ion $[M+HCOO]^-$ was the base peak, showing the $[M-H]^-$ ion as the most abundant fragment, except for *cis*-11-eicosenoic acid, which was detected as $[M-H]^-$ anion with a neutral loss of water to yield an $[M-H-18]^-$ ion.

A subclass of FA, acylcarnitines (CAR) was analysed using $[M+H]^+$ as precursor ion, showing their unique and informative MS/MS fragmentation pattern. Acylcarnitines dissociated to produce neutral losses of 59 and 161, as shown in Table 1.

Glycerolipids (GL), triacylglycerols (TAG) and diacylglycerols (DAG), could logically be placed in the same group as phospholipids (phosphatidylcholine, phosphatidylethanolamine, etc.) since both family members share a common glycerol backbone in their structure. However, these two types of lipids have entirely different chemical properties: neutral versus polar respectively [24]. The analysis of glycerolipids such as glyceryl trioleate and glyceryl tripalmitoleate, which have no electrostatic charge in solution, was carried out on the base of the molecular ion $[M+NH_4]^+$.

The most abundant MS/MS fragment ions correspond to the loss of one esterified fatty acyl group from the molecular ion as the free carboxylic acid, as well as ammonia, providing information about the fatty acyl groups of the TAG molecular species, as proposed by Murfy & Axersen in the TAG fragmentation scheme [24].

Glycerophospholipids (GP): phosphatidylcholines (PC) and phosphatidylglycerols (PG) were analysed, according to the polar head group of these molecules, with a preference for [M+H]+ ions for PC species, as shown in Table 1.

The fragmentation pattern for glycerophospholipids showed the characteristic fragment ions for each different class: m/z 184 for the phosphocholine family.

Ceramide (C18) belongs to the class of sphingolipids (SP), which includes structurally very different compounds. Table 1 shows molecular ions [M+H]+ used as precursors and the typical losses of H_2O to form [M−H_2O+H]+ ions (m/z 548 used as quantifier ion), with subsequent amide bond cleavage and neutral loss of the fatty acid to yield an ion with m/z 264 (qualifier).

Finally, for the sterol lipid family (ST) we considered the adduct with ammonium [M + NH_4]+. In our method we found the transition [M+NH_4]+ → [M+NH_4-35]+, considered to be the characteristic fragment for the quantification of this class of compounds, as shown in Table 1.

3.3. Validation

The method was validated for 33 lipids. The linearity range was evaluated using the R2 value, which was from 0.95 to 1.00. LOQ values were different for each compound and were in the range of 0.003 – 14.88 ngmL-1. The linearity data were used to assess the percentage of matrix effect (%ME), which was calculated as (1-slope in solvent/slope in matrix), expressed as a percentage [33,39]. The %ME values shown in Table 1 were in the range ±20, except for oleoyl-L-carnitine hydrochloride (-26%), palmitoyl-L-carnitine hydrochloride (-33.9%), lanosterol (-25.8%), *cis*-11-eicosenoic acid (-21.5%) and 1-monopalmitoleoyl-rac-glycerol (-20.8%). %ME in the range between -20% and 20% can be considered as insignificant because such variability is close to RSD repeatability values [39].

Intra-day and inter-day repeatability were evaluated for each lipid. CV % did not exceed a value of 15% for intra-day assay and 20% for inter-day assay (Table 3). The relative recovery ranges were over 90% for 12 compounds, between 80% and 90% for 8 compounds, between 70% and 80% for 5 compounds, between 60% and 70% for 6 compounds and below 60% for 1 compound. All these method validation results are summarised in Table 3.

None of the blank samples gave any signal that interfered with the peaks of the analytes after injection of the highest point in the calibration curve, showing no memory effect in the chromatographic run.

3.4 Quantification in real samples

One of the goals of this work was to demonstrate the feasibility of the analytical method developed for direct and rapid definition of lipid content in grape samples.

Table S1 summarises all the quantitative data for the 33 lipids quantified, expressed in terms of absolute amount (μg g^{-1} of fresh weight), measured in 18 healthy grape varieties (10 red and 8 white), harvested at technological maturity, in the same year (2010) and belonging to 4 different genetic groups: *Vitis vinifera*, *Vitis* non-*vinifera*, Muscat and hybrid [34]. We injected each extracted samples two times: non diluited and dilueted hundred times, in order to evaluate the most abundant lipids. The amounts are reported as μg g^{-1} of fresh sample after normalization, on the basis of the internal standard, docosahexaenoic acid. The most abundant compounds found were oleanolic acid, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine and glyceryltrioleate. In general, the amount of lipids was similar for all varieties, while we observed an increased amount of linoleic acid and oleic acid + *cis*-vaccenic acid in *vitis Champini* and Pinot noir. In Pinot noir we also found an increased amount of three fatty acids: linolenic acid, palmitic acid and *cis*-11-eicosanoic acid.

These preliminary observations suggest the existence of a certain diversity in the composition of grape lipids according to the cultivar, which requires more systematic confirmation. With regard to differences in the lipid profile or concentration linked to grape colour, we did not notice any particular trend for these samples. The wide genetic diversity of the grape samples chosen for this preliminary survey could have prevented the observation of minute differences inside red and white grapes belonging to a similar genetic group.

As regards the amount of lipids reported in the literature, it is difficult to make any comparison, because of the different varieties selected by other authors and because many papers have reported on the amount of lipids separately in tissue, skin, seed or pulp.

4. Conclusion

Due to their function, lipids are very important in biological systems. In plants in particular they have different roles and their relative abundance is subsequently influenced by genotype and phenotype. As knowledge of grape lipids is poor, a targeted LC-MS method for quantitative analysis of different classes of grape lipids was set up and optimized for the

extraction, identification and quantification of different classes of grape lipids: fatty acids, sterols, glycerolipids, glycerophospholipids and sphingolipids.

Specifically, the chromatographic method allowed us to obtain good separation of the different classes of lipids and the MRM method allowed us to be very specific, avoiding interference and analysing lipids after minimal sample preparation. The method described was used to produce a lipid profile for different grape varieties. It was successfully applied to the analysis of 18 grape samples (10 red grape and 8 white grape varieties) from 4 different genetic groups, with 33 lipids being identified and quantified, 23 of these being confirmed by the q/Q value. This protocol could be easily adapted to analyse other simpler grape products, such as grape must in particular. In conclusion, our method could be applied to the study of lipids both in whole grape samples and grape tissues. Moreover, the analysis of lipids in must during winemaking could be useful in order to better understand and exploit yeast metabolism, given the crucial importance of these compounds in the fermentation process [20,40,41].

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FIGURE 1

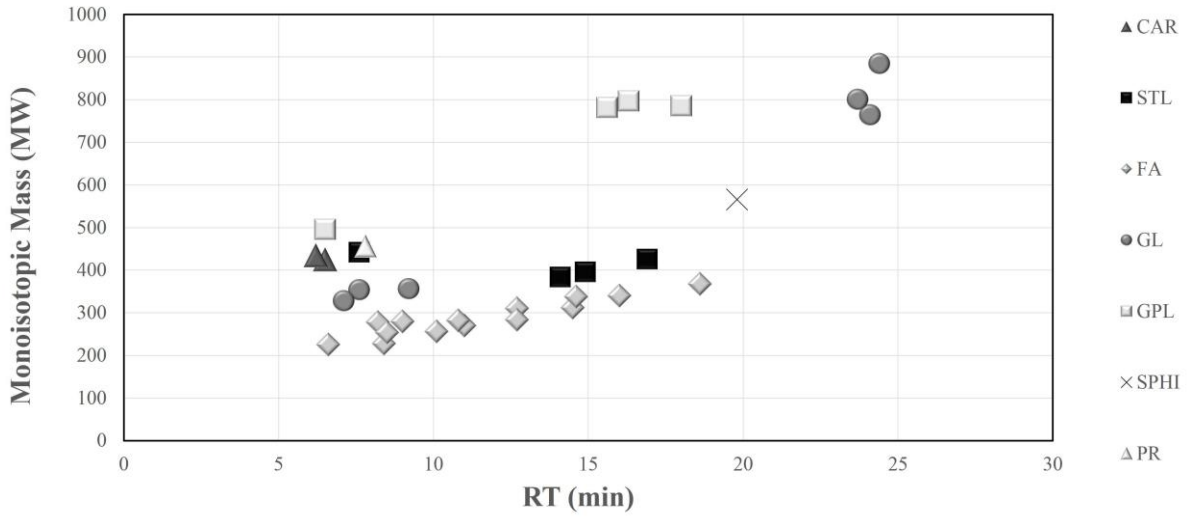
Monoisotopic Mass (MW)
1000
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
RT (min)
CAR
STL
FA
GL
GPL
SPHI
PR

FIGURE 2

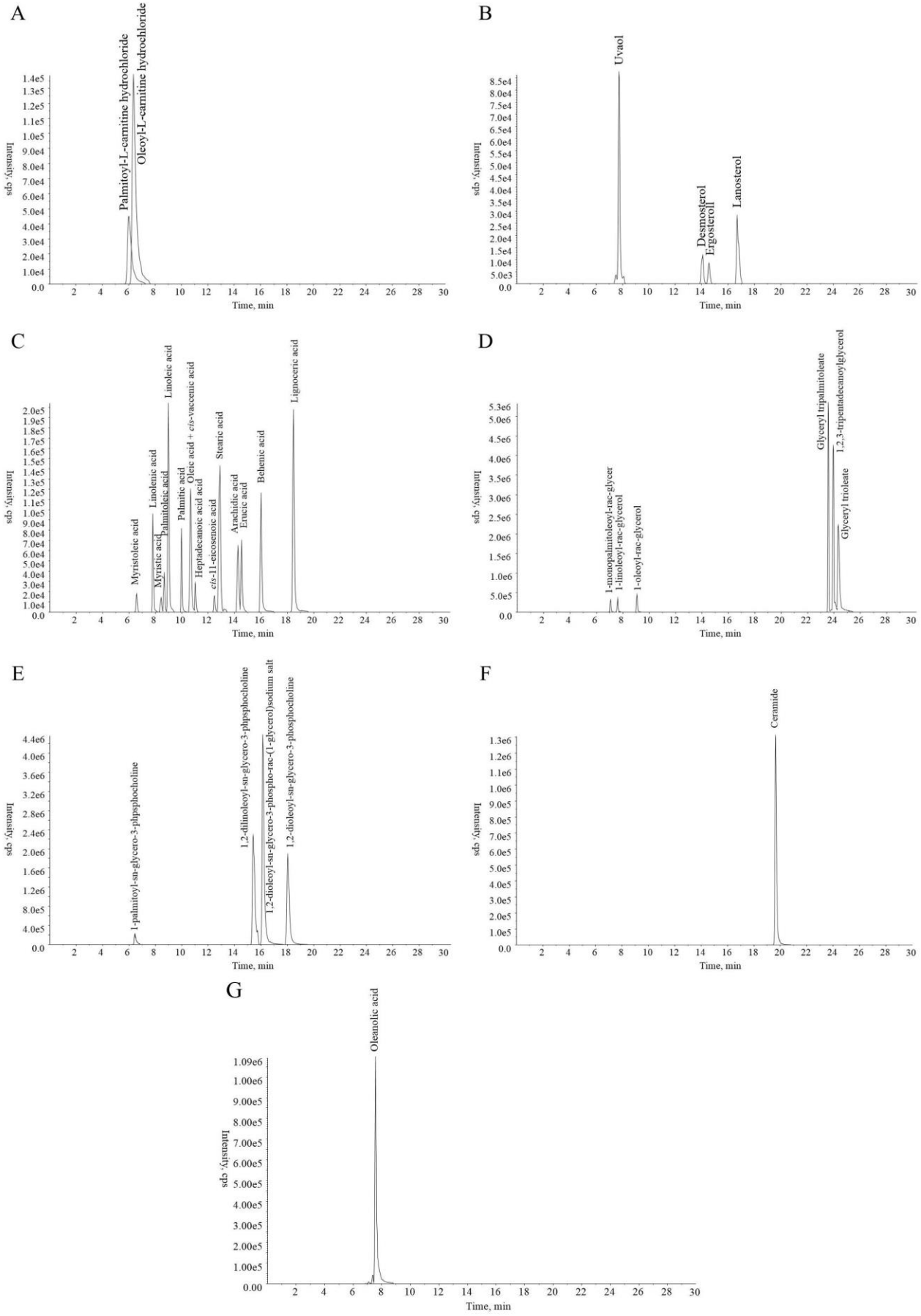
A
Palmitoyl-L-carnitine hydrochloride
Oleoyl-L-carnitine hydrochloride
Intensity, cps
Time, min
B
Uvaol
Desmosterol
Ergosterol
Lanosterol
Intensity, cps
Time, min
C
Myristoleic acid
Linolenic acid
Myristic acid
Palmitoleic acid
Palmitic acid
Heptadecanoic acid acid
Oleic acid + cis-vaccenic acid
cis-11-eicosenoic acid
Linoleic acid
Stearic acid
Arachidic acid
Erucic acid
Behenic acid
Lignoceric acid
Intensity, cps
Time, min
D
1-monopalmitoleoyl-rac-glycer
1-linoleoyl-rac-glycerol
1-oleoyl-rac-glycerol
Glyceryl tripalmitoleate
1,2,3-tripentadecanoylglycerol
Glyceryl trioleate
Intensity, cps
Time, min
E
1-palmitoyl-sn-glycero-3-plipsphocholine
1,2-dilinoleoyl-sn-glycero-3-plipsphocholine
1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt
1,2-dioleoyl-sn-glycero-3-phosphocholine
Intensity, cps
Time, min
F
Ceramide
Intensity, cps
Time, min
G
Oleanolic acid
Intensity, cps
Time, min

TABLE 1

Class	Compound	Linearity range (µg/mL)	Water calibration curves		Grape calibration curves		Matrix effect (%)	LOD (ng/mL)	LOQ (ng/mL)
			Equation	R^2	Equation	R^2			
Carnitines									
	Oleoyl-L-carnitine hydrochloride	0.0002-5.8	y=17816884x+1170800	1.00	y=14144865x+666169	1.00	-26.0	0.1	0.3
	Palmitoyl-L-carnitine hydrochloride	0.00002-4.36	y=39651340x+78003	1.00	y=29621992x+3023	1.00	-33.9	0.01	0.03
Sterols									
	Desmosterol	0.09-3.845	y=295822x+65554	0.95	y=274231x+108744	0.90	-7.9	19.3	57.9
	Ergosterol	0.09-1.985	y=100137x-12	0.99	y=87502x-3004	1.00	-14.4	49.6	148.8
	Lanosterol	0.05-1.0675	y=107149x-4434	1.00	y=85316x-4259	1.00	-25.8	21.3	63.9
	Uvaol	0.005-1.105	y=2507521x+86572	0.99	y=2295751x+49898	0.98	-9.2	2.2	6.7
Fatty acids									
	Arachidic acid	0.003-31.3	y=1971102x-83094	1.00	y=2046868x+264544	0.99	3.7	1.6	4.7
	Behenic acid	0.008-17.1	y=2607531x+130259	1.00	y=2628503x-52941	1.00	0.8	3.4	10.2
	cis-11-eicosenoic acid	0.07-15.5	y=7673x+1005	0.99	y=6317x+1060	0.99	-21.5	31.1	93.3
	Erucic acid	0.001-33.9	y=4874309x-636823	0.99	y=4949227x-656680	0.99	1.9	0.8	2.5
	Heptadecanoic acid	0.01-27.1	y=531131x+728127	0.99	y=486268x+460828	0.99	-9.2	11.3	33.9
	Lignoceric acid	0.003-9.2	y=2712524x+683788	0.98	y=2841008x+337484	0.98	4.5	1.9	5.6
	Linoleic acid	0.007-28.0	y=2563516x+5753986	0.97	y=2170119x+8184061	0.94	-18.1	7.0	21.0
	Linolenic acid	0.006-69.5	y=821382x+828911	0.99	y=842024x+806833	0.99	2.5	2.8	8.4
	Myristic acid	0.05-57	y=365484x+96999	1.00	y=334746x+185754	1.00	-9.2	22.8	68.4
	Myristoleic acid	0.05-56.5	y=391868x-15488	1.00	y=368574x-36527	1.00	-6.3	11.3	33.9
	Oleic acid + cis-Vaccenic acid	0.02-28.3	y=1975170x+9760208	0.96	y=1777976x+3723146	0.97	-11.1	14.1	42.3
	Palmitic acid	0.1-32	y=19724x+12835	1.00	y=17856x-19368	0.98	-10.5	32.0	128.2
	Palmitoleic acid	0.02-25.4	y=601474x+285142	0.99	y=534259x+284641	0.99	-12.6	12.7	38.1
	Stearic acid	0.002-28.4	y=1476286x+1248339	0.99	y=1506209x-142367	0.99	2.0	1.4	4.3
Glicerolipids									
	1.2.3-tripentadecanoylglycerol	0.00007-0.8	y=119296649x+6912932	0.96	y=120666830x+3263178	0.97	1.1	0.04	0.1
	1-linoleoyl-rac-glycerol	0.02-14.2	y=139154x+1410	1.00	y=116890x-10601	0.97	-19.1	14.2	42.6
	1-monopalmitoleoyl-rac-glycerol	0.02-52.5	y=1270229x-135275	1.00	y=1051850x-109119	1.00	-20.8	10.5	31.5
	1-oleoyl-rac-glycerol	0.003-17.9	y=1951698x-242429	1.00	y=1799361x-506911	1.00	-8.5	1.8	5.3
	Glyceryl trioleate	0.03-7.1	y=7274665x+2401909	0.96	y=6816032x+1467346	0.97	6.7	14.1	42.3
	Glyceryl tripalmitoleate	0.003-3.5	y=34292391x+1504691	1.00	y=35000163x-39599	1.00	2.0	1.4	4.3
Glicerophospholipids									
	1.2-dilinoleoyl-sn-glycero-3-phosphocholine	0.01-15.6	y=59249626x+21763259	0.98	y=58952735x+19159586	0.98	-0.5	6.3	18.8
	1.2-dioleoyl-sn-glycero-3-phosphocholine	0.006-12.6	y=60549785x+10796759	0.99	y=63008040x+11502248	0.99	3.9	3.1	9.4

	1.2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt	0.01-12.8	y=41360769x+1615101	0.99	y=41001805x+3811341	0.97	-0.9	6.4	19.2
	1-palmitoyl-sn-glycero-3-phosphocholine	0.01-19.9	y=14155899x+678383	1.00	y=11959790x+2361447	0.99	-18.4	8.0	23.9
Sfingolipids									
	Ceramide	0.0002-14.9	y=14453621x+5731780	0.97	y=15128604x+6550053	0.96	4.5	0.1	0.4
Prenols									
	Oleanolic acid	0.04-4.6	y=1604598x+474133	0.97	y=1469483x+592518	0.95	-9.2	22.8	68.4

TABLE 2

Class	Compound	Ionization Mode	Precursor ion	Q1					Q2					RT	Q2/Q1
				Product ion	DP	EP	CE	CXP	Product ion	DP	EP	CE	CXP		
Carnitines															
	Oleoyl-L-carnitine hydrochloride	[M+H]+	426,3	367,0	120	11	26	17	265,0	120	11	30	13	6,5	39,0
	Palmitoyl-L-carnitine hydrochloride	[M+H]+	400,3	341,0	173	10	24	14	239,0	173	10	28	14	6,2	33,8
Sterols															
	Cholesterol (IS)	[M+1-H2O]+	369,5	147,0	140	8	35	25	135,0	140	8	35	23	15,7	0,6
	Desmosterol	[M+NH4]+	402,4	367,2	146	10	13	20						14,1	
	Ergosterol	[M+H]+	397,3	69,3	6	10	33	14	379,4	6	10	17	22	14,9	150,1
	Lanosterol	[M+NH4]+	444,3	409,5	51	10	13	32	191,3	51	10	21	16	16,9	38,3
	Uvaol	[M+NH4]+	460,4	443,4	100	10	11	22	191,4	100	10	21	10	7,6	40,5
Fatty acids															
	Arachidic acid	[M+HCOO]-	357,2	311,4	-5	-10	-12	-21	45,1	-5	-10	-40	-5	14,5	46,7
	Behenic acid	[M+HCOO]-	385,4	339,4	-60	-11	-15	-10	321,4	-60	-11	-47	-16	16,0	0,3
	cis-11-eicosenoic acid	[M-H]-	309,4	291,5	- 167	-11	- 32,9	-19	155,0	- 167	-11	-37	-10	12,7	16,2
	Docosahexaenoic acid (IS)	[M+HCOO]-	373,3	327,3	-60	-10	-10	-21	283,4	-60	-10	-20	-20	8,4	0,1
	Erucic acid	[M+HCOO]-	383,2	337,3	-95	-10	-12	-23	45,0	-95	-10	-44	-5	14,6	35,6
	Heptadecanoic acid	[M+HCOO]-	315,4	269,3	-45	-13	-10	-17	45,0	-10	-10	-32	-5	11,0	64,6
	Lignoceric acid	[M+HCOO]-	413,2	367,3	-65	-10	-12	-23	45,0	-65	-10	-42	-5	18,6	35,9
	Linoleic acid	[M+HCOO]-	325,2	279,2	-60	-12	-10	-21	261,0	-60	-12	-35	-23	9,0	0,4
	Linolenic acid	[M+HCOO]-	323,3	277,2	-65	-6	-8	-17	259,3	-65	-6	-27	-17	8,2	0,5
	Myristic acid	[M+HCOO]-	273,2	227,2	-5	-10	-18	-15	45,0	-5	-10	-26	-7	8,4	99,2
	Myristoleic acid	[M+HCOO]-	271,3	225,3	-45	-11	-20	-17	45,0	-30	-10	-26	-7	6,6	87,6
	Oleic acid + cis-Vaccenic acid	[M+HCOO]-	327,2	281,2	-50	-10	-14	-17	45,1	-50	-10	-34	-5	10,8	48,4
	Palmitic acid	[M+HCOO]-	301,2	255,3	-55	-10	-16	-23	44,9	-55	-10	-38	-5	10,1	80,2
	Palmitoleic acid	[M+HCOO]-	299,3	253,3	-55	-10	-18	-15	44,7	-55	-10	-34	-7	8,5	37,6
	Stearic acid	[M+HCOO]-	329,3	283,3	-60	-12	-9	-22	265,5	-60	-12	-41	-16	12,7	0,3
Glicerolipids															
	1,2,3-tripentadecanoylglycerol	[M+NH4]+	782,7	523,5	106	4	31	33	225,4	106	4	49	17	24,1	6,5
	1-linoleoyl-rac-glycerol	[M+H]+	355,3	337,0	300	3	15	16	263,0	300	3	13	21	7,6	25,0

1-monopalmitoleoyl-rac-glycerol	[M+H]+	329,3	121,2	104	7	29	9	219,3	104	7	17,5	17	7,1	179,7
1-oleoyl-rac-glycerol	[M+H]+	357,3	265,2	250	10	15	13	283,2	250	10	13	17	9,2	27,2
Glyceryl trioleate	[M+NH4]+	902,8	265,0	80	8	55	14	603,0	80	8	60	14	24,4	59,8
Glyceryl tripalmitoleate	[M+NH4]+	818,7	547,0	300	4	28	27	237,3	300	4	53	12	23,7	22,3
Glicerophospholipids														
1,2-dilinoleoyl-sn-glycero-3-phosphocholine	[M+H]+	782,6	184,0	100	15	27	23	125,0	100	15	123	35	15,6	78,7
1,2-dioleoyl-sn-glycero-3-phosphocholine	[M+H]+	786,6	184,0	116	3	27	14	125,0	116	3	134	14	18,0	57,6
1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt	[M-Na+NH4]+	792,5	603,6	50	6	35	15	339,3	50	6	45	14	16,3	2,9
1-palmitoyl-sn-glycero-3-phosphocholine	[M+H]+	496,4	478,4	100	14	27	14	184,0	100	14	35	14	6,5	641,5
Sfingolipids														
Ceramide	[M+H]+	566,6	548,6	100	8	18	14	264,0	100	8	41	14	19,8	54,7
Prenols														
Oleanolic acid	[M+NH4]+	474,3	439,4	146	10	15	32	191,2	146	10	27	12	7,8	27,7

TABLE 3

Class	Compound	Intra-day CV (%)	Inter-day CV (%)	Recovery	Accuracy	Precision
Carnitines						
	Oleoyl-L-carnitine hydrochloride	1,40	14,28	88,77	-11,23	3,07
	Palmitoyl-L-carnitine hydrochloride	2,49	13,49	83,29	-16,71	4,12
Sterols						
	Desmosterol	5,21	17,41	60,04	-39,96	20,14
	Ergosterol	3,22	7,93	82,73	-17,27	41,81
	Lanosterol	3,99	19,89	95,86	-4,14	11,52
	Uvaol	3,23	17,52	115,71	15,71	6,13
Fatty acids						
	Arachidic acid	3,10	1,23	63,39	-36,61	5,34
	Behenic acid	2,65	4,44	67,38	-32,62	9,75
	cis-11-eicosenoic acid	6,97	1,47	105,47	5,47	6,39
	Erucic acid	2,70	3,66	68,89	-31,11	3,19
	Heptadecanoic acid	3,89	1,91	82,79	-17,21	6,23
	Lignoceric acid	2,62	6,56	66,29	-33,71	5,10
	Linoleic acid	2,82	6,27	92,24	-7,76	9,63
	Linolenic acid	2,83	2,97	99,25	-0,75	8,05
	Myristic acid	3,22	2,94	79,02	-20,98	3,76
	Myristoleic acid	2,88	6,35	78,87	-21,13	5,18
	Oleic acid + cis-Vaccenic acid	3,45	3,56	79,84	-20,16	33,15
	Palmitic acid	18,86	20,35	84,74	-15,26	3,36
	Palmitoleic acid	2,23	3,82	92,25	-7,75	4,42
	Stearic acid	5,11	2,61	72,48	-27,52	14,38
Glicerolipids						
	1,2,3-tripentadecanoylglycerol	0,96	5,23	80,63	-19,37	6,80
	1-linoleoyl-rac-glycerol	4,56	13,47	95,73	-4,27	11,26
	1-monopalmitoleoyl-rac-glycerol	3,07	5,27	82,06	-17,94	5,86
	1-oleoyl-rac-glycerol	2,71	3,95	92,01	-7,99	5,82
	Glyceryl trioleate	1,07	8,89	32,54	-67,46	1,99
	Glyceryl tripalmitoleate	0,76	4,80	71,35	-28,65	3,04
Glicerophospholipids						
	1,2-dilinoleoyl-sn-glycero-3-phosphocholine	1,48	6,50	82,01	-17,99	5,68
	1,2-dioleoyl-sn-glycero-3-phosphocholine	1,33	2,39	64,82	-35,18	7,33
	1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)sodium salt	1,55	6,33	90,37	-9,63	1,82
	1-palmitoyl-sn-glycero-3-phosphocholine	1,76	1,97	91,68	-8,32	4,01
Sfingolipids						
	Ceramide	1,12	8,14	124,14	24,14	4,89
Prenols						
	Oleanolic acid	2,59	14,56	153,54	53,54	3,39