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# New insights in the composition of Wax and Sterol Esters in common and mutant sunflower oils revealed by ESI-MS/MS.

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**Abstract**

Wax esters (WEs) and steryl esters (SEs) are minor components of sunflower oils formed by the esterification of long chain fatty alcohols and sterols to fatty acids. These compounds have similar carbon numbers and polarities making them difficult to separate using conventional chromatographic methods. In this study, electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) allowed the rapid and accurate profiling of WEs and SEs acyl moieties in total ester fractions of common and mutant sunflower oils with different fatty acid profiles. The acyl composition of both WEs and SEs partially reflected that of the oil and the high oleic background displayed the lowest level of crystallisable waxes responsible for oil turbidity. ESI-MS/MS complemented by GC-MS analyses revealed that SEs contain 17-30 % of previously unreported methylsterol moieties. We demonstrated that these compounds are overlooked by official sterol analytical methods which may have consequences for quality control and authentication of vegetable oils prior to commercialisation.

*Keywords*: Wax esters; sterol, esters, sunflower oil, ESI-MS/MS, high oleic, turbidity, methylsterols

## 1. Introduction

Sunflower oil is one of the most widely used seed oils (Salas et al, 2015). This oil is extracted from sunflower seeds and is composed of triacylglycerols (TAGs) plus minor components that confer additional nutritional value, such as tocopherols and phytosterols (Verleyen et al., 2002; Velasco et al., 2002). Among the minor components that can be found in sunflower oil, waxes have special importance from a technological point of view. Wax esters (WEs) are molecules resulting from the esterification of a fatty acid and a long chain fatty alcohol. These compounds are found on the surface of many plant organs undertaking a protective function in association with hydrocarbons and other components (Kunst & Samuels, 2003). Wax is extracted together with the oil and is thus present as a minor component after oil refining. However, sunflower WEs are linear and often highly saturated molecules that generally display higher melting points than TAGs (Ruiz-Lopez et al., 2017). This cause the crystallization of the saturated wax species at room temperature (18-24$^{o}$C), conferring an undesirable turbidity to the oil. Thus, sunflower oil must be totally or partially dewaxed before retailing. Oil dewaxing is classically carried out by winterization and filter aid filtration. Crystallization involves the slow cooling of the oil from its cloud point temperatures down to a final temperature of 2 to 10 $^{o}$C to allow the formation of larger crystals. Crystallized waxes are then filtered after addition of filter aids (Dijkstra, 2015). The whole process takes several hours and involves the loss of a part of the oil with the filter aid, increasing the processing costs of sunflower oil.

In addition to common sunflower lines (*e.g*. CAS 6) a number of mutant lines with altered oil fatty acid compositions have been developed. These lines display a range of phenotypes such as high oleic acid (CAS 9), high stearic-high linoleic acids (CAS 3) and a high oleic-high palmitic acids (CAS 12). High oleic (HO) sunflower lines were first reported by Soldatov (1976) and their oil has been commercialised since the late 90´s. The high stearic-high palmitic lines have been developed by breeding and mutagenesis of common lines and can be an important alternative to oil palm in the future (Fernández-Moya et al., 2005; Martínez-Force et al., 1999). These lines have certain steps of the lipid biosynthetic pathway altered. These

alterations do not only affect triacylglycerides but also other lipid classes (Salas et al., 2006; Aznar-Moreno et al. 2014) so it would be of additional interest if they had altered WEs and/or SEs compositions.

The wax composition of sunflower oil has been a controversial topic within the field of oil analysis and characterization. Carelli et al. (2002) separated the waxes from the sunflower TAGs by liquid chromatography and analysed the resulting wax fraction, which showed high levels of linoleate and oleate in the fatty acid moieties and a primacy of saturated species (C18 to C24) in the fatty alcohols fraction. Other authors analysed waxes filtered in the dewaxing process, which displayed different composition, predominantly saturated species of fatty acids and fatty alcohols (Kanya et al., 2007). The analysis of waxes in vegetable oils is hampered by the high number of other compounds that co-elute with them during purification by normal phase liquid chromatography on silica gel. Thus, steryl esters (SEs), which are the most abundant minor components in sunflower oil, were frequently extracted and derivatised with WEs altering the results of quantitave and qualitative analysis of these compounds by gas chromatography. To solve this problem Henon et al. (2001) proposed a method based on LC using argented silica gel. This method removes most of the compounds interfering with the analysis of WEs by GC but involved the calculation and use of response factors that makes the analysis long and tedious. An improvement to this method, which includes the use of an internal standard, was later reported (Carelli et al., 2012). In this method, the problem of the possible retention of unsaturated WEs on the argented silica gel, which could lead to their underestimation, was studied. Thus, waxes containing one oleic acid moiety were recovered with over 90% efficiency, but no data was given about polyunsaturated waxes, which are also present in sunflower WEs (Carelli et al., 2002).

Free and esterified sterols are also minor compounds of interest in vegetable oils are. Phytosterols have been reported to have beneficial effects on human health because they help control the level of cholesterol associated to low density lipoproteins (Oslund, 2007). Moreover, knowing the exact sterol composition of vegetable oils is of interest to detect fraud in some oil blends (Cercaci et al. 2003; Bell & Gillat; Azadmard-Damirchi et al. 2010). The total sterol composition of sunflower oil is well known and was first reported by Itoh et al. (1973a). The main components were reported to be campesterol, stigmasterol, β-sitosterol, Δ5-

and Δ7-avenasterols and Δ7-stigmastenol. A similar sterol composition was reported for the Steryl ester fraction of sunflower oil (Phillips et al., 2002; Verleyen et al., 2002). However, the few studies published to date reported only the proportion of SEs in sunflower oil and the sterol composition of this fraction after hydrolysis by saponification, but no information is available about the fatty acid content and the molecular species composition.

The aim of the present work was to reveal the precise acyl-chain composition of WEs and SEs in sunflower oils using ESI-MS/MS to overcome the difficulties encountered with common chromatographic methods. The total amounts of wax in sunflower oil can vary significantly due to differences in the genetic background of the seed lines used, the kernel/hull ratio or the extraction method. For these reasons, the aim of this study was not to quantify WEs and SEs in sunflower oil but instead focussed on elucidating the molecular species composition of these two classes of compounds. Here we report the detailed composition and structure of WE and SE species in a common sunflower line and show differences in the composition of these classes of compounds in mutant lines. We also detected substantial amounts of methylsterols (C30+) recently overlooked in sunflower oil. Complementary GC-MS analysis suggested that these include the C30 4-monomethylsterols obtusifoliol and citrostadienol and the C31 4,4-dimethylsterol 24-methylenecycloartenol. The consequences of these results on the analytical treatment of minor components in vegetable oils are discussed.

## 2. Experimental

### *2.1 Plant material and oil extraction*

Sunflower plants of the four lines studied were grown in a glass house equipped with fertirrigation lines at 25◦/15◦C (day/night) with a 16 h photoperiod and a photon flux density of 300 µmol$m^{-2}$ $s^{-1}$. Multiple plants were cultivated for each line at random positions and with sufficient space between the plants to avoid shading, so environmental conditions were considered homogenous. Once seeds were developed irrigation was suppressed and allowed to dry to a final humidity content of less than 10%. Oil was extracted from sunflower whole seeds using a Sohxlett apparatus. Paper cartridges containing 20g of ground dry seeds

(approx.) and the same weight of anhydrous sodium sulphate were made and loaded into the Sohxletts. Oil was extracted with hexane for 24h and then the solvent was removed by distillation in rotavapor. The resulting crude oils were used for WE and SE determinations. Oil extractions from each line were obtained from multiple independent plants with the subsequent statistical analyses based on three or four biological replicates as indicated in figure legends.

*2.2 Solid phase extraction of WE and SE*

WE and SE were isolated from the oil matrix by solid phase extraction. Amounts of 0.2 g of oil were dissolved in 1 mL of elution solvent (hexane/ethyl ether 99:1), and loaded onto a Discovery SPE DSC-Si Silica Tube extraction cartridge (20 mL-5g, Supelco) previously activated at 100°C for at least 2h and equilibrated in the same solvent. The cartridges were eluted with 35 mL of the same solvent and fractions of 5 mL were collected. Typically, WE and SE were eluted free of TAGs in the interval between 15 and 20 mL of elution. The composition of each fraction was checked by thin layer chromatography on Lichrospher HPTLC silica gel 60 F254S plates (Merk), which were developed with hexane/ethyl ether 90:10. Fractions containing the purified WE and SE were pooled and concentrated under $N_2$ without drying the samples. The purity of these total ester (TE) fractions was checked again by HPLC analysis (AOCS Official Method Cd 11d-96), then they were submitted to MS studies.

2.3 ESI-MS/MS analysis of WEs and SEs

The method used for ESI-MS/MS analyses of WEs was adapted essentially from Iven et al. (2013). Analysis of WE and SE was performed with an Applied Biosystems 4000 QTRAP Triple quadrupole mass spectrometer with a linear ion trap and an electrospray ionisation source (ABSciex, USA) operated in positive ionisation mode and using a direct infusion setup. Ester compounds were monitored via multiple-reaction monitoring (MRM) using mass transitions optimized for sensitivity and selectivity of both WEs and SEs molecular species. Ammonium adducts $[M+NH_4]^+$ were selected as precursor ions with $[RCO_2H_2]^+$ selected as the product ion for WE analysis, or $[M-(RCOOH+NH3)]^+$ as the product ions for SE analysis, and with R corresponding to the fatty acid moietie. The ion pairs used for WE monitoring can be found in Iven

et al. (2013). To improve signal intensity, a range of wax-ester standards were optimised using the Analyst software (AB Sciex, USA), which allowed optimisation of declustering potential, entrance potential, collision energy and determination of collision exit potential values which could then be assigned to structurally related wax-esters. The ion pairs used for SE monitoring were adapted from Wewer et al. (2011) and are described in supp. Table 1.

Underivatized WEs from TE fractions were initially separated and visualised by GLC as described in Ruiz-lopez et al. (2017) (data not shown). For ESI-MS/MS analysis TE samples were dried under nitrogen and re-suspended in 24:24:2 v/v/v chloroform/methanol/250 mM ammonium acetate buffer containing 6 WE internal standards (ISs; C12:0/C14:0, C14:0/C16:0, C17:0/C17:0, C17:1/C17:0, C17:0/C17:1 and C17:1/C17:1 at a final concentration of 1.21, 0.92, 0.81, 0.67, 1.74 and 1.27 pmol/µl respectively). These ISs were used to account for differential ionisation and fragmentation due to acyl chain length and/or unsaturation. All samples were centrifuged prior to injection to pellet any particulates and were then directly infused into the mass spectrometer at a rate of 10 µl/min using a PAL-HTS-xt (CTC, Switzerland). Tubing was flushed after each sample, and the line purged with sample for 2.5 minutes prior to data acquisition. The MRM scan was run for 4.2 minutes, with an initial 15 second equilibration period prior to the acquisition, and peak intensities were averaged over the entire data acquisition period. A 20 second negative ion mode run followed each sample to maintain signal intensity and reduce quadrapole charging effects. The voltage across the probe was 5.5 kV with a nebulizing gas pressure of 20 psig, a curtain gas pressure of 15 psig and a collision cell pressure of 4 psig. The turbo gas pressure and temperature were set to off, and the CEM was set to 2.3 kV. QTRAP data was analysed initially using the LipidView software (AB Sciex, USA) to identify MRM transitions, and the data was exported to Microsoft Excel for further processing.

Wax ester ESI-MS/MS data were normalised sequentially firstly to account for type 1 isotopic distribution, and then for differential responses associated with compound structural features, essentially chain length, degree of desaturation and distribution of double bonds between fatty acyl and fatty alcohol moieties. This was achieved by calculating correction factors using the internal standards to generate linear regressions for each sample. This method was described in detail by Brügger et al. (1997). The Sterol esters ESI MS/MS

data generated in this study were used essentially for comparative analyses to investigate compositional differences in SE acyl moieties between CAS6 and the mutant lines. This was achieved by expressing the signal intensity measured for each SE compound detected as a percentage of total SE signal intensity. Semi-quantitative analysis of sterol in oil and SE fractions was carried out using gas chromatography as described below.

*2.4 Sterol determination in seed oil (total) and SE fractions using GC-FID and GC-MS*

To determine seed total sterol content and composition 100 mg of de-hulled seeds was crushed and the homogenate was extracted twice with 1 ml of hexane for 30 minutes at 38°C. Aliquots of both the seed total lipid and the TE samples were dried under nitrogen, saponified and silylated using standard procedures (Winkler-Moser J. 2011). After derivatization, sterols were quantified by GC-FID (HP6890 Series) using cholestanol as an internal standard and identified using a GC-MS instrument (Agilent 6890N GC-5975B MS system). Both GC systems operated using identical columns and oven conditions. A HP1-MS capillary column (Agilent J&W; 30m, 0.25mm, 0.25µm) was used with splitless injection, Helium as carrier gas with a flow rate of 2ml/min and the inlet temperature set at 325°C. The oven temperature program started at 200°C and was ramped to 325°C at the rate of 6.5°C per minute, with the final temperature held for a further 4 minutes. Both the FID and the MSD transfer line temperatures were set at 325°C. The MS source and the quadrupole temperatures were set at 230°C and 150°C, respectively, and the m/z scan range was set between 42 and 520. Sterol identity was assigned using the NIST mass spectral search program and database, and an in-house database constructed using authentic standards and validated oil samples. Each compound's identity was confirmed using the retention indices method and authentic standards (Cholesterol, campesterol, β-sitosterol and lanosterol), and sterols retention times and mass spectra available in the literature for further validation.

*2.5 Statistical analyses*

To test for compositional differences between common CAS 6 and mutant sunflower lines, a one-way ANOVA was applied to each compound. A logit transformation was first applied to these data to ensure homoscedasticity. For compounds below the level of detection in a particular sample or line, a nominal

value of 0.01 was assigned. For each set of tests (i.e. for each figure), a Benjamini-Hochberg (B-H) adjustment was applied to all compounds detected (giving the number of responses) to control the false discovery rate of the analysis. Comparisons to CAS6 were deemed noteworthy if i) the one-way ANOVA on 8 residual degrees of freedom was statistically significant after a B-H correction ($p_{BH} < 0.05$) and ii) the difference in abundance on the logit scale was greater than the B-H adjusted least significant difference ($LSD_{BH}$). In a small number of cases we further investigated the data through specific contrasts for individual compounds. In order to preserve the controlled false discovery rate, the Benjamini-Hochberg adjustment calculated from the set of one-way ANOVAs was further applied to the associated structured ANOVA with line specific contrasts (as detailed through the RFM approach of Hassall and Mead (2018))

A principal components analysis was applied to the sterol composition of oil (total sterol) and SEs in CAS 6. Due to the compositional structure of the data, values of zero were replaced by 0.01 subject to the trace replacement method of (Aitchison, 1986). Moreover, data were analysed on the centred log ratio scale given by, $z_i = log\left(\frac{y_i}{\prod_{j=1}^{D} y_j}\right)$, where $y_i$ is the percentage composition of compound *i,* for compounds *i* = 1,…,*D* (Aitchison, 1986). Thus, the interpretation of the multivariate analysis should be seen as the relative effect of each compound compared to the geometric mean.

All statistical analyses were done using the statistical software package R (version 3.4.2).

**3. Results and discussion**

The composition of waxes in sunflower oil has been a matter of debate for decades because WEs are difficult to separate from the much more abundant SEs using simple silica-based chromatographic methods. Several alternative methods, e.g. based on argentation chromatography (Henon et al., 2001; Carelli et al., 2012), have been described to separate these two classes of compounds however, these are quite difficult and labour intensive. MS-based methods offer greater sensitivity for detecting minor components as well as providing structural information, including molecular weights (MW) and mass spectra, which together with chromatographic data allow much more reliable compound identification. GC-MS methods, however, are limited to volatile compounds and require complicated purification and

derivatisation procedures for analysis of complex biological samples. In recent years, ESI-MS has emerged as a sensitive and reliable technique for analysing non-volatile or thermally labile bio-molecules that are not amenable to analysis by other conventional techniques (Griffiths, 2003; Banerjee and Mazumdar 2012). Softer ionisation methods and the additional separation capabilities of tandem mass spectrometry provide powerful and sensitive tools for the detection and the structural elucidation of bio-molecules sharing similar molecular structures, in complex mixtures (Ho et al. 2003; Haslam and Feussner, 2017). Targeted analyses using direct infusion and MRM methods allow further simplification of the analytical procedure and rapid profiling of molecular species without the requirement for advanced compound purification and elaborate mass spectra interpretation (Iven et al., 2003).

3.1 Wax ester composition of sunflower oils

Crude seed oils extracted from different lines of sunflower were used in this study (oil compositions in supp. Table 2). A previous separation from TAGs by solid phase extraction was necessary to obtain total ester (TE) fractions containing WEs and SEs that were analysed by ESI-MS/MS as described in methods. We detected over 50 molecular species but WEs were initially grouped according to their carbon chain length and degree of desaturation to facilitate comparison with previous reports of sunflower oil wax composition (Figure 1). The WEs identified ranged from 32 to 48 carbons comprising saturated, monounsaturated and di-unsaturated molecular species. In agreement with most previous reports, the majority of the WEs detected in CAS 6 ranged from 36 to 48 carbons, however, substantial amounts of C32 and C34 species were also detected. This took on greater significance as the proportion of these shorter compounds increased dramatically in all the mutant lines analysed in this study. Total sunflower WEs were distributed in two groups with very distinct molecular species compositions (Figure 1). The first group contained shorter chains (C32-C38) comprised of over 70% unsaturated molecular species whereas the second group contained longer chains (C40-C48) with ca. 70% saturated WEs (Sup. Table 3). The most abundant WEs in common sunflower (CAS6) were 34:1, 36:1 and 36:2 in the first group, and 42:0, 44:0 and 46:0 in the second but with substantial amounts of other very long chain WEs (i.e. 46:1, 48:0 and 48:1). These two

groups of compounds also appeared in the other mutant lines investigated; although the percentage of very long chain mostly saturated WEs (C40+, Group 2) were significantly different between lines, with lower levels detected in the high oleic line (CAS 9) which, instead, displayed elevated amounts of saturated and monounsaturated C34 and C36 WEs (Group 1) (sup. Table 4 and Supp figure 1). Interestingly, levels of monounsaturated C40-C44 WEs appeared unaffected in the same line. A similar effect, although less pronounced, was observed in the high stearic high linoleic line (CAS 3), whereas the high palmitic high oleic-mutant line (CAS 12) displayed a profile specially depleted in three of the highest carbon number species C46:1, C48:0 and C48:1. Small amounts of longer WEs (up to C52) have been reported in sunflower, as well as the presence of odd chained derivatives (Carelli et al., 2002). However, only traces of these WEs were present in the oils analysed here and these compounds did not generate sufficient signal intensity for reliable determination by ESI-MS/MS. This was confirmed by GC-MS analysis using Single Ion Monitoring (SIM) acquisition methods, which revealed minute amounts of C41 and C43 WE co-migrating with isoprenoid degradation products (data not shown). These compounds were most likely derived from EI degradation of the much more abundant SEs present in the TE fractions analysed. This hypothesis is consistent with a later report by Carelli et al. showing that those compounds are no longer present in WE fractions after removal of SEs by chromatographic separation on silver nitrate-impregnated silica gel (Carelli et al. 2012).

These groups of WEs are formed by different fatty acid and fatty alcohol moieties as illustrated in Figure 2. In CAS 6 the composition of the abundant C34 and C36 WEs in the first group was dominated by 16:0, 18:0 and 18:1 species in both the fatty acid and alcohol moieties and the most abundant molecular species all contained oleic acid (i.e. 16:0/18:1, 18:0/18:1 and 18:1/18:1). In the second group, the composition of the major C42 to C46 WEs was dominated by molecular species containing 20:0, 22:0 and 22:1 fatty acids in combinations with 22:0, 24:0 and 26:0 fatty alcohols and the most abundant molecular species (>5% of total WE) contained eicosanoic acid (i.e. 22:0/20:0, 24:0/20:0 and 26:0/20:0) or erucic acid (24:0/22:1). Surprisingly, only two minor species contained linoleic acid (16:0/18:2 and 18:0/18:2). The higher level of C34 and C36 WEs observed in high oleic CAS 9 oil resulted from an increased proportion of molecular

species containing either 16:0, 18:0 or 18:1 fatty alcohols esterified with a 18:0 or C18:1 fatty acids (16:0/18:0, 16:0/18:1, 18:0/18/0, 18:1/18:0, 18:0/18:1 and 18:1/18:1), at the expense of saturated very long chain species containing C20, C22 and C24 fatty acids. The most abundant molecular species remained 16:0/18:1 and 18:0/18:1, albeit increased more than 2-fold in CAS 9 compared to common sunflower CAS 6 (supp figure 2). The levels of monounsaturated C40-C44 WEs in CAS 9, which were unaffected, were explained by higher levels of molecular species containing oleic acid (i.e. 22:0/18:1, 24:0/18:1 and 26:0/18:1), compensating for the decreases of isomeric species containing very long chain fatty acids. Surprisingly, the higher levels of unsaturated C34 and C36 WEs observed in the high stearic high linoleic CAS 3 line was due to a dramatic increase in species containing an 18:1 alcohol moiety (18:1/16:0, 18:1/18:0 and 18:1/18:1). Particularly, the increase in 36:2 was not due to the high 18:2 fatty acid content of that line. All these observations were found to be statistically significant (supp table 2, supp figure 2). Finally, the high palmitic-high oleic CAS 12 line displayed higher contents of species containing 16:0 (18:0/16:0 and 16:0/16:0) and 16:1 (16:0/16:1, 18:0/16:1 and 20:0/16:1) fatty acid moieties in the first group of long chain WE. The second very long chain WE group was characterised by increased levels of species containing oleic acid, as observed in CAS 9, but lower levels of species containing fatty acids longer than C20.

Published evidence suggests that longer WEs (C40+) are produced in the hull while the shorter species seem to be more closely associated with the seed kernel (Carelli et al, 2002). The presence of molecular species containing erucic acid in the second group of waxes (e.g. 24:0/22:1 and 26:0/22:1), which represent nearly 10 % of total WE acyl moieties in CAS 6 (Figure 2), support this hypothesis since this fatty acid was not detected in CAS 6 kernels (Salas et al. 2005) and must therefore be produced in the hull. This is significant from a technological point of view because longer chained saturated WEs, which display higher melting points, crystallize at room temperature conferring turbidity to the oil. For this reason, longer saturated WEs are sometimes referred to as crystallisable waxes. For instance, the two most abundant WE species in the first group C34:1 (16:0/18:1) and C36:1 (18:0/18:1) have melting point temperatures of 18 and 24 °C, respectively, whereas C40:0 (20:0/20:0 and 22:0/18:0) the shortest WE species present in the

second group have a melting points temperature of ca. 70 °C (Ruiz-Lopez et al., 2017). Generally, all mutant lines displayed a decreased proportion of WE from the second group but the most dramatic effect was observed in the high oleic line CAS 9, which displayed a 50% reduction in very long chain WEs compared to common sunflower CAS 6 (C40-C48, Supp. Table 4, supp figure 1B). This considerably decreased proportion of saturated crystallisable waxes in CAS 9 may be expected to yield an oil with less turbidity than common sunflower varieties. CAS 3 also displayed a partial reduction in C40-C48 WEs however this mainly affected monounsaturated species (i.e. C40:1-C46:1) which have lower melting points while saturated species appeared less significantly affected (supp figure 2A). The slight reduction in very long chain WEs observed in CAS 12 was specifically due to decreases in three minor molecular species, two of them being mono unsaturated (i.e. 46:1, 48:0 and 48:1).

*3.2 Fatty acid and fatty alcohol composition of sunflower oils wax esters*

The total fatty acid composition of this fraction was extracted from the ESI-MS/MS profiling data obtained for each molecular species. As shown in Figure 3A 18:1 and 20:0 were the most abundant acyl moieties in common sunflower oil wax, which also contained around 10% of each 18:0, 22:0 and 22:1. Interestingly, 18:2 represented less than 5% of total fatty acids in WEs in contrast with a previous study reporting linoleate as the major fatty acid in sunflower oil wax (Carelli et al., 2002), presumably due to co-extraction with SEs acyl moieties. The CAS 9 high oleic line displayed higher proportion of 18:0 and 18:1 fatty acids at the expense of 18:2 and very long chained fatty acids (figure 3A, supp Figure 3). The high stearic high linoleic CAS 3 line displayed higher contents of 16:0 and 18:1 and, similarly to CAS 9, lower amount of very long chain fatty acids. As discussed above, 18:2 appears to be very inefficiently incorporated in the sunflower seed wax ester fraction despite an elevated background in this line. The high palmitic high oleic line CAS12 displayed a fatty acid profile similar to CAS 3 but with slightly increased 16:1 and an unaffected level of 20:0. With regard to fatty alcohols, the profile corresponding to the common sunflower line displayed mostly saturated moieties with the exception of 10% 18:1 and small percentages of 20:1 and 26:1

(Figure 3B). The predominant moieties were 18:0, 22:0, 24:0, and 26:0 with contents ranging between 10 and 20% of total fatty alcohol. The high oleic line displayed higher contents of 16:0 and 18:1 fatty alcohol at expenses of longer moieties (supp figure 4). The high stearic line-high linoleic CAS 3 also displayed lower levels of very long chain alcohol moieties but differed from the other three lines analysed in this study due to an elevated content in oleoyl alcohol in its wax ester fraction, which was 3-fold higher than the control line CAS6 (Supp figure 4). The high palmitic-high oleic line displayed a profile of fatty alcohols comparable to common sunflower for the major molecular species.

Comparing common sunflower with mutant lines revealed that changes in the fatty acid background affected the composition of both groups of WEs, albeit not always as might have been predicted based on FA composition in the oil (Figure 3; supp. table 2). For instance, the elevated content in oleic acid in CAS 9 resulted in an increase in the proportion of this fatty acids in both the acyl and the fatty alcohol moieties of WEs, however, a lower content of the same fatty acid in CAS 3 did not have the expected reciprocal effect. Instead CAS 3 WEs displayed levels of 18:1 acyl moieties comparable to CAS 9 and, surprisingly, a 3-fold increase in oleoyl alcohol. Similarly, linoleic acid which represents over 48 % and 43 % of total fatty acid in CAS 6 and CAS 3 oils, respectively (Supp. table 2; Fernandez-Moya et al. 2003), appeared almost completely excluded from WEs representing less than 5% of total acyl moieties in both lines. This is in sharp contrast to a previous report, which indicated that 18:2 was the major fatty acid in sunflower WEs accounting for 44 % of the total acyl moieties (Carelli et al, 2002).

*3.3 Sterol and fatty acid composition of sunflower oil SEs*

Phytosterol are known to occur mainly as free sterols (FS) and esterified forms in all vegetable oils. Sunflower oil was shown to contain 30-40% phytosterol in the SE form but little information is available in the literature about their sterol composition and even less about their acyl moieties (Verleyen et al., 2002). SEs were analysed by ESI-MS-MS to gain knowledge about their molecular species composition and, in particular, investigate the effect of variation in oil fatty acid composition on acyl moieties in this fraction. The molecular weight of the sterol-derived product ions detected in sunflower SEs ranged from 381.4 to

409.4 Da corresponding to C28 to C30 sterol moieties with rounded MW ranging from 398 to 426 Da (supp. Table 1). As illustrated in Figure 4 (box inserts in each panel) the total signal intensity detected was similar between lines for most of the five groups of compound masses detected, suggesting that the overall composition of the sterol moieties in SEs is not significantly different between common sunflower CAS 6 and the 3 mutant lines analysed in the study. This was confirmed by statistical analyses with the possible exception of the 400 Da compounds which appeared elevated in CAS9 and CAS 12 (Supp. Figure 5). It should be noted that for most molecular masses detected the signal intensities probably derived from two or three sterol isomeric forms e.g. campesterol and $\Delta$7-ergostenol (MW 400) or $\beta$-sitosterol and D7-stigmastenol (MW 414) as illustrated later by GC-MS analyses (supp. Table 5). Interestingly, a strong signal was obtained for compounds with a molecular mass of 426 Da (C30 sterol) suggesting the presence of substantial amounts of methylsterols, either 4-monomethyl- or 4,4-dimethylsterols, in this fraction. Analysis of the acyl moieties of SEs revealed a contrasting picture with different fatty acid profiles for the line analysed compared with WEs (Figure 4, main panels). For all SE detected the predominant sterol-bonded acyl chains were 18:1 and/or 18:2, with lower amounts of 16:0, 16:1 and 18:0 and only traces of 18:3. SEs based on C30 sterols moieties (MW 426) also contained a small proportion of 20:0, 22:0 and 24:0 very long chain fatty acids (VLCFAs), which were otherwise completely excluded from other SEs species. Lines with a high linoleic background, CAS 6 and CAS 3 displayed a high proportion of SEs carrying 18:2 as the acyl moiety, whereas lines with a high oleic background (CAS 9 and CAS 12) contained 18:1 as the predominant SE acyl species. The high palmitic line CAS 12 also displayed higher proportions of 16:0 and 16:1, particularly in SEs containing a 414 or 426 Da sterol moiety. This comparative analysis of SE acyl composition was carried out with 4 biological repeat to increase to statistical significance of the data generated (supp table 10, sup figure 5). The total fatty acid composition of total SE fractions displayed a similar pattern to individual SE groups for each sunflower line (Figure 5) with oleic and linoleic acids representing between 70-90% of total acyl moieties in all lines. Specifically, 18:2 accounted for 62% of the fatty acids in the common CAS 6 line and 68% in the high stearic-high linoleic CAS 3 line. In contrast, in the high oleic lines CAS 9 and CAS 12 18:1 represented ca. 68% and 63% of total acyl moieties, respectively.

Other fatty acids accounted for lower proportions, in all cases below 15 %, despite a two-fold increase in 16:0 and 16:1 in CAS12. Johansson & Appleqvist (1979) reported that sunflower oil SEs contain a high percentage of long chain saturated fatty acids, however, our work demonstrated that this fraction is in fact almost completely depleted from VLCFAs and instead mainly composed of esters of oleic and linoleic acids in the common sunflower CAS 6 line. Generally, the acyl composition of SEs reflected well the oil total fatty acid composition in the CAS 3, CAS 6 and CAS 9 lines analysed (Figure 5; Supp. table 1). Only the acyl species derived from C30 methylsterols (MW 426) displayed a slightly higher proportion of saturated fatty acids including traces of VLCFAs (Figure 4).

To complement the structural data obtained by ESI-MS/MS, the detailed sterol composition of SEs was determined for each sunflower line by GC-MS and GC-FID after saponification of TE samples. This allowed the identification of ten main sterol species in the SEs (Figure 6A). A further four minor sterol and triterpenoid peaks were detected by GC but since these compounds each represented less than 1% of the total molecular species detected, and some of them contained more than one compound (See below), these peaks were not integrated (Supp. Table 5). Consistent with the ESI-MS/MS analyses there was no major difference in desmethylsterol composition between common sunflower CAS 6 and other mutant lines. In all lines the predominant sterol species were β-sitosterol and $\Delta^7$-stigmastenol each accounting for 20 to 30% of the sterols in the oil SE fractions analysed. GC-MS analyses also confirmed the presence of a significant amount of methylsterols which, combined, represented ca. 17-30% of the total sterol moieties in sunflower oil SEs (Figure 6A). These included the 4-monomethyl C30 sterols obtusifoliol and citrostadienol (MW 426) and the 4,4-dimethyl C31 sterol 24-methylenecycloartanol (MW 440). A small amount of graminasterol, a 4-monomethyl C29 sterol, was also detected co-migrating with β-amyrin just before the $\Delta^7$-stigmastenol peak on GC chromatograms (Data not shown, Supp. Table 5). The identity of these methylsterols was assigned based on chromatographic and MS data and validated by comparison with those available in the literature for sunflower and other vegetable oil total sterols. However, these identities remain to be unequivocally confirmed using identical molecular standard. Among other sterols identified $\Delta^7$-Avenasterol was found at around 10% and the other species, Campesterol, $\Delta^5$-avenasterol,

Δ7-ergostenol and Stigmasterol represented 5% of total sterol moieties or lower. Although no correlation with fatty acid content and no clear pattern of variation could be established for sterol composition between the lines analysed, CAS 3 SEs a to contain a higher percentage of obtusifoliol whereas CAS 9 and CAS 12 lines SEs appeared depleted from this sterol moiety compared to CAS 6. These differences proved to be statistically significant (supp table 12, supp figure 7). CAS 9 also displayed a lower proportion of $\Delta^7$-Avenasterol than other lines but this was not found significant in our experimental conditions.

We detected and identified many more sterol moieties than previously reported for sunflower SE composition (Johansson & Appleqvist, 1979; Phillips et al. 2002; Verleyen et al., 2002) but the most remarkable difference compared with all previous studies was the presence of C30 and C31 methylsterols representing ca. 25% of total SEs in CAS6 oil (Figure 6A). These 4-methylsterols and 4,4'-dimethylsterols are metabolic precursors of the 4-desmethylsterols end products of plant sterol biosynthetic pathways, and thus are usually present at lower levels in mature plant tissues (Winkler-Moser, AOCS Lipid Library). A possible reason for this difference is discussed below. The minorC28 (MW 398) compound detected by ESI-MS/MS (Figure 4) could not be resolved by GC with the experimental conditions used in this study and probably comigrated with a more abundant compound. In contrast with the fatty acyl moieties the distribution of sterol species in SEs was only moderately affected by variations in total fatty acids composition and all mutant sunflower lines analysed displayed similar profiles to the common sunflower line CAS6 (Figure 6A).

*3.4 Total sterol composition of sunflower oil*

Since the presence of 4-monomethyl- and 4,4-dimethylsterols in sunflower oil was previously only reported after saponification in purified methylsterol fractions separated from 4-desmethylsterols on TLC (Fedeli et al., 1966; Kornfeldt, & Croon, 1981; Itoh et al. 1973b), or more recently in oil total sterol extracts (Roche et al. 2010a and b), we wished to compare the composition of these sterols in SEs with the oil total sterol composition. As shown in Figure 6B for Common sunflower CAS6, SEs displayed a 50% reduction in the proportions of the C29 4-desmethylsterols β-sitosterol and stigmasterol compensated by increased levels

of several of their precursors, including $\Delta^7$-avenasterol but also the C30 4-monomethylsterols citrostadienol and obtusifoliol. The proportion of $\Delta^7$-stigmastenol also increased in SEs but other sterols showed more modest differences. These compositional differences were investigated by PCA analysis which suggested that steryl esters are associated with high relative abundance of $\Delta$7-Ergosterol, Obtusifoliol, $\Delta$7-stigmastenol, $\Delta$7-avenasterol and citrostadinienol, whilst oil total sterol is associated with a high relative abundance of campesterol, sitosterol and stigmasterol (sup figure 8). Decreases in $\beta$-sitosterol and sigmasterol accompanied by increased levels of $\Delta^7$-avenasterol and $\Delta^7$-stigmastenol in sunflower oil SEs compared to free sterols are consistent with previously published work (Johansson & Appleqvist 1979, Phillips et al. 2002) but enrichments of sunflower SEs in methylsterols has never been reported before. Intrigued by the absence of methylsterols in most recent studies reporting the sterol lipids composition of sunflower oil we decided to investigate the possible reasons for these discrepancies. Oil total sterol chromatograms obtained using the method described in the experimental section were compared with that obtained using the standard method for sterol preparation and analysis from vegetable oils (ISO 12228-1:2014). Remarkably, the standard preparation (Supp. Figure 9B) lacked the peaks corresponding to C30 obtusifoliol, citrostadienol and C31 methylenecycloartanol sterol species although, combined, these represented ca 15% of total sterols and 25% of SEs in the sunflower oil preparations analysed in this study (Figure 6B).

The occurrence of these compounds in many vegetable oils, including sunflower, has long been known (Itoh et al, 1973b; Kornfeldt & Croon, 1981; Roche et al. 2010a and b) and they were shown to be particularly abundant in olive and sunflower oils (Fedeli et al., 1966). However, methylsterols are missing in most recent studies reporting the sterol lipid composition of sunflower oil (Phillips et al. 2002; Verleyen et al., 2002; Rosa a et al., 2009; Grompone, 2011; Li et al., 2011; Aguirre et al, 2012; Nestola & Schmidt, 2016) and their presence, distribution and enrichment in the SE fraction of any oil has never been reported before. Before the elucidation of phytosterols biosynthetic pathways and the identification of methylsterols as precursors of desmethylsterols, these compounds were often described as triterpene alcohols that were recovered as a separate band on TLC plates together with $\alpha$ and $\beta$-amyrin, lupeol and butyrospermol (Itoh

et al, 1973b; Fedeli et al., 1966). Chromatographic separation steps were included in official standard methods and recommended practices for the analysis of sterol content and composition in fats and oils, including the more recent ISO norm 12228. Therefore, it is likely that only the 4-desmethylsterol fraction was often collected and analysed while methylsterols were generally discarded together with triterpenoid compounds. This was demonstrated in this work by comparing the composition of total sterols from CAS 6 oil isolated without and with the separation of unsaponifiable compounds on TLC before silylation and GC analysis (Supp. Figure 9). Clearly the C30 and C31 methylsterols (peak 6, 14 and 15) were not recovered with desmethylsterols in the band extracted from TLC (Supp. Figure 9B) which resulted in biased total sterol analysis. This is of special significance since sterol composition is used for quality control and authentication of vegetable oils, but also to detect adulteration and fraudulent blends of expensive edible oils, such as olive oil, with oils of lower quality or cheaper seed oils (e.g. Cercaci et al. 2003). Furthermore, it was suggested that methylsterols provide better markers for these purposes since they vary more among vegetable oils (Azadmard-Damirchi, 2010). These controls were made possible by the standardisation of sterol profiling procedures and analytical data of oils is often required for commercialisation. In light of these results, modifications of the standard methods should be considered to allow quantification of 4-monomethyl- and 4,4'-dimethylsterol sterol species and their inclusion in the official standard composition of sunflower and other vegetable oils.

**4. Conclusions**

In this study, ESI-MS/MS allowed the rapid and accurate profiling of WE and SE acyl moieties in complex total ester fractions of common and mutant sunflower seed oils, alleviating the requirement for laborious purification procedures. This is the first report of how altered fatty acid compositions in sunflower mutants alter the composition of oil minor lipid fractions such as SEs and WEs.

Total WEs appeared distributed across two distinct groups containing either C32 to C38 mostly unsaturated or C40 to C48 mainly saturated molecular species. The former resulted from the esterification of fatty acids commonly found in sunflower oil, whereas the latter, formed by VLCFAs believed to be produced in the

hull, may represent the crystallisable wax responsible for sunflower oil turbidity. High oleic CAS 9 displayed a 50% reduction in very long chain saturated waxes and, amongst the lines analysed in this study, presents the greatest potential for developing a crystallisable wax-free sunflower oil that would help reducing processing costs. Changes in the fatty acid background affected the composition of both groups of WEs, although in some lines these changes did not reflect the oil composition. In constrast with previous reports, all lines displayed low levels (<5%) of C18:2 acyl moieties in WEs.

In all lines, SEs displayed similar sterol compositions whereas acyl moieties were dominated by either oleic or linoleic acid depending on the FA background. Saturated FAs represented less than 20% of acyl chains in SEs and VLCFAs were almost completely excluded from these compounds. CAS6 SEs displayed reduced levels of C29 phytosterol end products, compared to oil total sterols, and were instead enriched in sterol biosynthetic precursors, including C30 and C31 methylsterols moieties previously unreported in sunflower. Despite representing 17-30 % of total SEs in the sunflower lines analysed in this study, we demonstrated that these compounds are overlooked by official sterol analytical methods. This may have consequences for quality control and authentication of vegetable oils prior to commercialisation.

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**Figure legends**

**Figure 1.** Wax ester composition of oils extracted from different sunflower lines. CAS 6: common sunflower (■), CAS 3: high stearic-high linoleic (■), CAS9: high oleic (■), CAS 12: high palmitic-high oleic (☐). Bars shown are the average of 3 biological replicates plus minus standard error. Compounds showing significant differences across all lines are denoted by * corresponding to a one-way ANOVA p-value adjusted for multiplicity of <0.05 (shown in supp table 6 and illustrated in sup. Figure 1A and B). For 40:0, 42:0, 44:0 and 46:0 applying a structured ANOVA comprising of a set of orthogonal contrasts revealed evidence of statistical differences. These contrast specific differences are marked with • (F statistic and p values are given in supp Figure 1C)

**Figure 2.** Wax ester molecular species composition of oils extracted from different sunflower lines. CAS 6: common sunflower (■), CAS 3: high stearic-high linoleic (■), CAS9: high oleic (■), CAS 12: high palmitic-high oleic (☐). Bars shown are the average of 3 biological replicates plus minus standard error. Compounds showing significant differences across all lines are denoted by * or ** corresponding to a one-way ANOVA p-value adjusted for multiplicity of <0.05 or <0.001 (shown in supp table 7 and sup. Figure 2A). For 18:0/18:1, 24:0/20:0 and 26:0/20:0 applying a structured ANOVA comprising of a set of orthogonal contrasts revealed evidence of statistical differences. These contrast specific differences are marked with • (F statistic and p values are given in supp Figure 2B)

**Figure 3.** Fatty acid (A) and fatty alcohol (B) composition of wax esters in oils extracted from different sunflower lines. CAS 6: common sunflower (■), CAS 3: high stearic-high linoleic (■), CAS9: high oleic (■), CAS 12: high palmitic-high oleic (☐). Bars shown are the average of 3 biological replicates plus minus standard error. Compounds showing significant differences across all lines are denoted by * or ** corresponding to a one-way ANOVA p-value adjusted for multiplicity of <0.05 or <0.001 (shown in supp tables 8 and 9 and illustrated in supp Figures 3A and 4A). For 18:1 FA, 20:0 FA and 20:0 FA-OH applying a structured ANOVA comprising of a set of orthogonal contrasts revealed evidence of statistical differences.

These contrast specific differences are marked with • (F statistic and p values are given in supp Figures 3B and 4B)

**Figure 4.** ESI-MS/MS analysis of the molecular species composition of SEs in oils from different sunflower lines. Inserts: Total ion signal intensities for each sterol moiety detected. The Carbon number and nominal mass of the sterol moiety is given (MW). Main panels: Composition of the fatty acid moieties associated with each sterol type identified. CAS 6: common sunflower (■), CAS 3: high stearic-high linoleic (■), CAS9: high oleic (■), CAS 12: high palmitic-high oleic (□). Bars shown are the average of 4 biological replicates plus minus standard error. Compounds showing significant differences across all lines are denoted by * or ** corresponding to a one-way ANOVA p-value adjusted for multiplicity of <0.05 or <0.001 (shown in supp table 10 and illustrated in supp Figure 5).

**Figure 5.** Total Fatty acid composition of SEs in oils extracted from different sunflower lines. CAS 6: common sunflower (■), CAS 3: high stearic-high linoleic (■), CAS9: high oleic (■), CAS 12: high palmitic-high oleic (□). Bars shown are the average of 4 biological replicates plus minus standard error. Compounds showing significant differences across all lines are denoted by ** corresponding to a one-way ANOVA p-value adjusted for multiplicity of <0.001 (shown in supp table 11 and illustrated in supp Figure 6).

**Figure 6.** GC analysis of the sterol composition of sunflower oil SE and total oil. (A) Sterol composition of SE from different sunflower lines. CAS 6: common sunflower (■), CAS 3: high stearic-high linoleic (■), CAS9: high oleic (■), CAS 12: high palmitic-high oleic (□). Compounds showing significant differences across all lines are denoted by ** corresponding to a one-way ANOVA p-value adjusted for multiplicity of <0.001 (shown in supp table 12 and illustrated in supp Figure 7). (B) Comparison of the sterol composition in CAS6 oil SEs and total oil.. Compounds denoted by ♦ correspond to a higher relative abundance in either total sterol or SEs as revealed by PCA analysis (shown in supp table 13 and illustrated in supp Figure 8). Bars shown are the average of 3 biological replicates plus minus standard error.

Figure 1

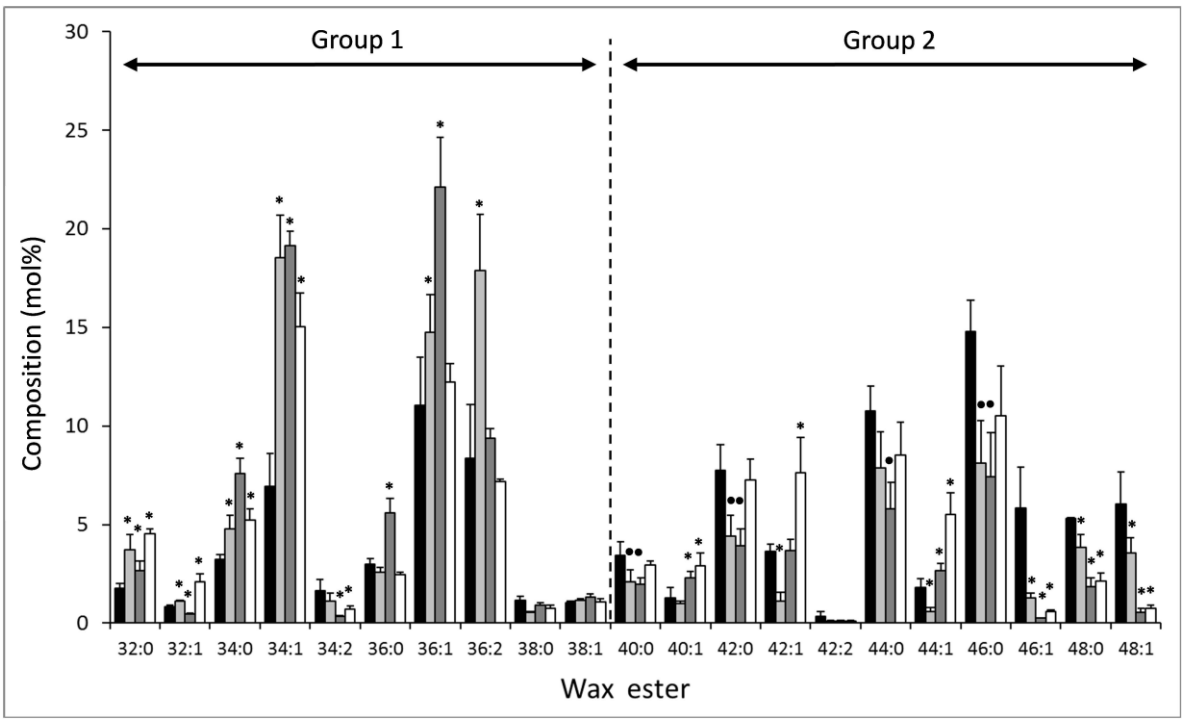

Group 1
Group 2
Composition (mol%)
30
25
20
15
10
5
0
32:0
32:1
34:0
34:1
34:2
36:0
36:1
36:2
38:0
38:1
40:0
40:1
42:0
42:1
42:2
44:0
44:1
46:0
46:1
48:0
48:1
Wax ester

Figure 2

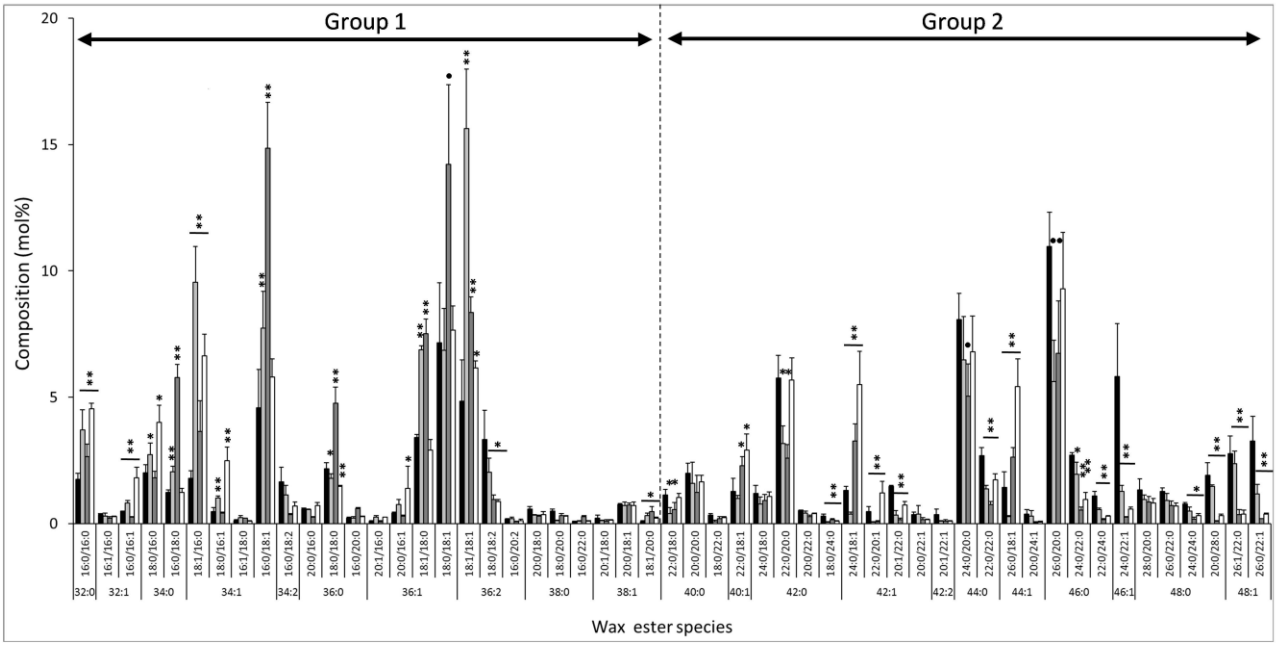


Figure 3

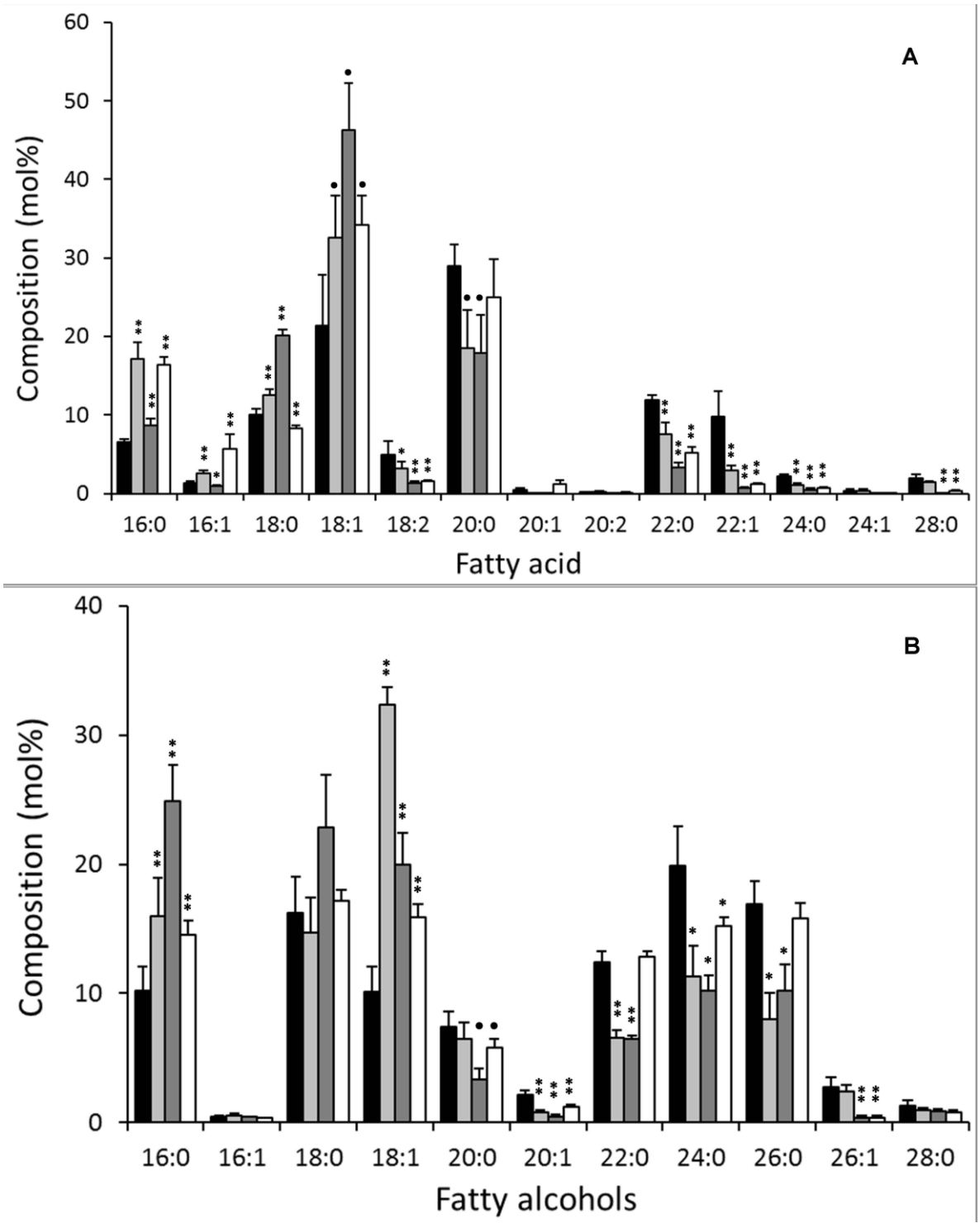

A
Composition (mol%)
60
50
40
30
20
10
0
16:0
16:1
18:0
18:1
18:2
20:0
20:1
20:2
22:0
22:1
24:0
24:1
28:0
Fatty acid
B
Composition (mol%)
40
30
20
10
0
16:0
16:1
18:0
18:1
20:0
20:1
22:0
24:0
26:0
26:1
28:0
Fatty alcohols

Figure 4

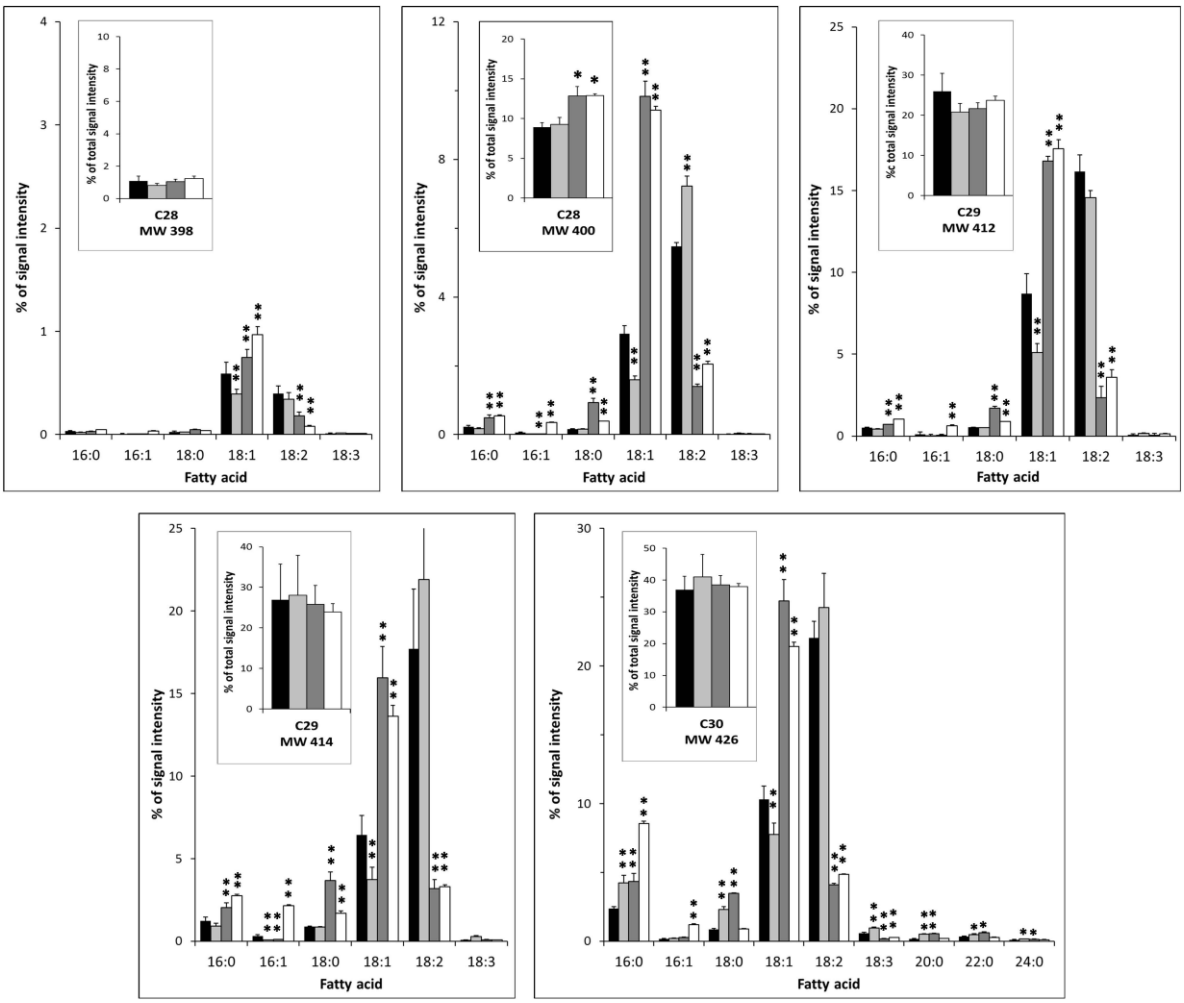

% of signal intensity
Fatty acid
16:0
16:1
18:0
18:1
18:2
18:3
% of total signal intensity
C28
MW 398
C28
MW 400
C29
MW 412
C29
MW 414
C30
MW 426
20:0
22:0
24:0

Figure 5

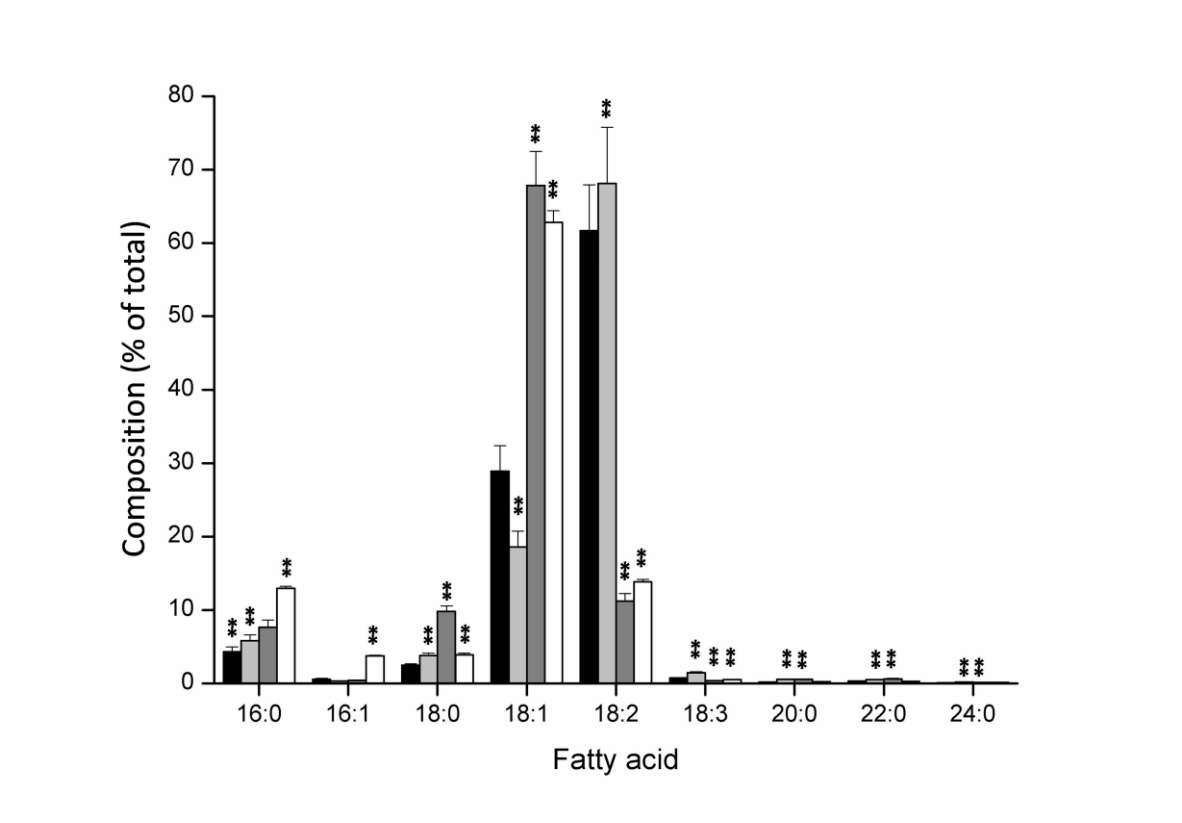
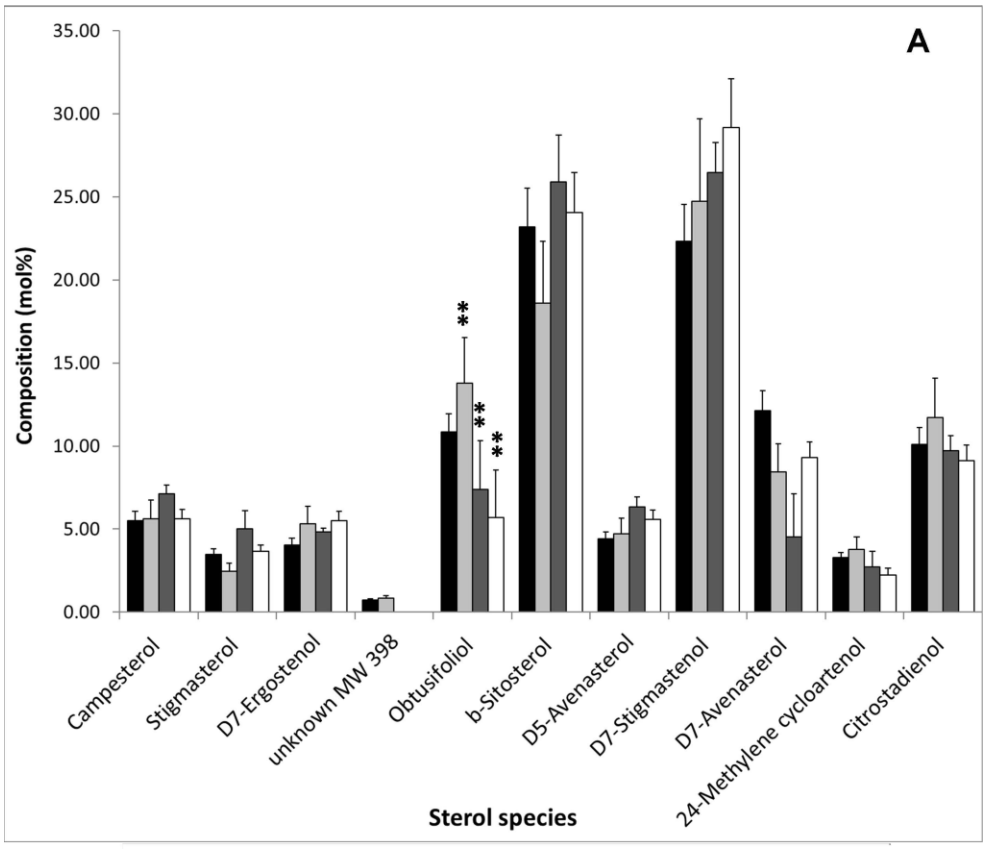
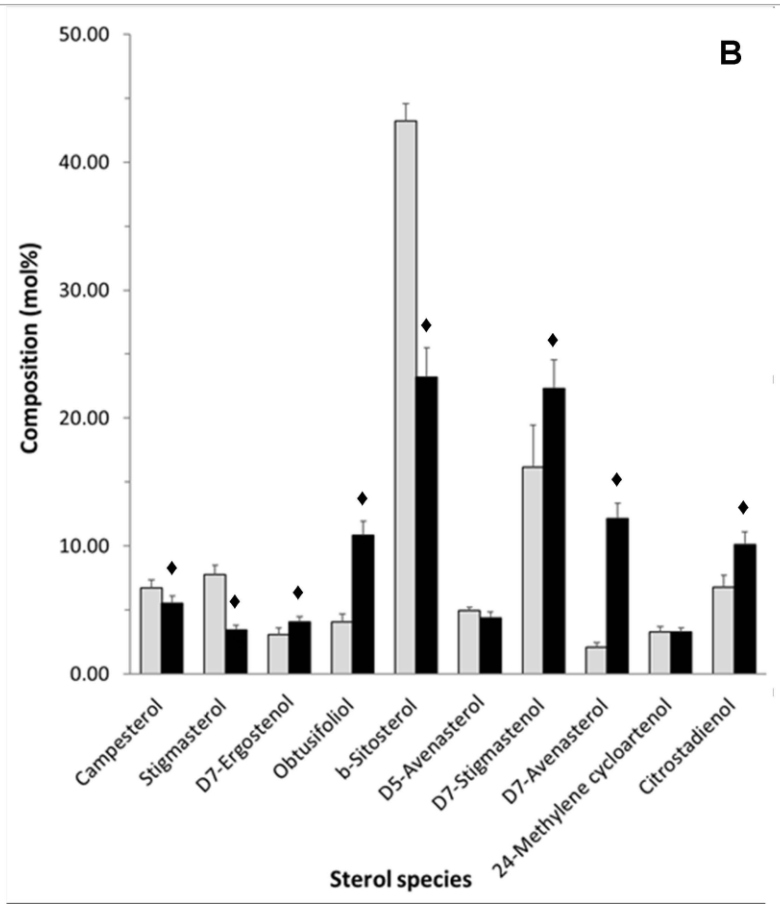

Composition (% of total)
80
70
60
50
40
30
20
10
0
16:0
16:1
18:0
18:1
18:2
18:3
20:0
22:0
24:0
Fatty acid

Figure 6


A
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Composition (mol%)
**
**
**
Campesterol
Stigmasterol
D7-Ergostenol
unknown MW 398
Obtusifoliol
b-Sitosterol
D5-Avenasterol
D7-Stigmastenol
D7-Avenasterol
24-Methylene cycloartenol
Citrostadienol
Sterol species


B
50.00
40.00
30.00
20.00
10.00
0.00
Composition (mol%)
♦
Campesterol
Stigmasterol
D7-Ergostenol
Obtusifoliol
b-Sitosterol
D5-Avenasterol
D7-Stigmastenol
D7-Avenasterol
24-Methylene cycloartenol
Citrostadienol
Sterol species