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Rapid Identification of Triacylglycerol-Estolides in Plant and Fungal Oils.

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Abstract

Triacylglycerol-estolides are components of the storage oil of certain plant and fungal species and are generally associated with the presence of fatty acids containing hydroxyl groups. These unusual acyl-glycerols can easily go undetected when oils are analysed by techniques such as gas chromatography of fatty acid methyl esters, or thin layer chromatography. We describe the detection of TAG-estolides in seeds of *Lesquerella lyrata* and sclerotia of the ergot fungus *Claviceps purpurea* using ^{1}H MAS-NMR for non destructive analysis. We also conducted analysis of small amounts of oil by MALDI-TOF MS to clearly show the presence of TAG-estolides and to rapidly characterize their acyl composition. The matrix used in this work was 2,4,6-trihydroxyacetophenone (THAP) made up in sodium chloride-saturated solvent. We were able to confirm the presence of TAG-estolides with no free hydroxyl groups in the fungal oil, and TAG-estolides with free hydroxyl groups in the oil of *Lesquerella lyrata*. The development of a technique for the rapid identification of TAG-estolides in oil samples will simplify the detection of these novel lipids in plant and fungal species.

Key words. Estolide, *Lesquerella fendleri*, *Lesquerella lyrata*, Ergot (*Claviceps purpurea*), Seed oil, Hydroxy fatty acids.

Abbreviations

FAMES	Fatty acid methyl esters
HFA	Hydroxy fatty acid
MALDI-TOF- MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
AG	Acylglycerol
DAG	Diacylglycerol
TAG	Triacylglycerol

1. Introduction

An estolides is an acyl ester in which the carboxylic acid group of a fatty acid forms an ester linkage with either a hydroxyl group or a double bond in a second fatty acid. Synthetic estolides, formed from monounsaturated or hydroxy fatty acids by chemical reaction (Achaya, 1971; Erhan et al., 1992), or by lipase catalysis (Hayes and Kleiman, 1995; Bodalo-Santoyo et al., 2005) have shown considerable promise as base stocks for biodegradable lubricants and in the manufacture of coatings and cosmetics (Cermak and Isbell, 2004; Isbell et al., 2001). Their properties are a factor of the fatty acid components, and the average number of fatty acids linked together. In addition to fatty acids, estolides have also been successfully synthesized by esterifying additional fatty acids to the hydroxy fatty acid moieties of triacylglycerol (TAG) oils that naturally contain these unusual fatty acids (Isbell et al., 2002; 2006). The resulting TAG-estolides have novel properties conferred by their branched structure. Estolide and TAG-estolide based functional fluids are predicted to have a considerable advantage over purely triacylglycerol based products due to the greater hydrolytic stability of the secondary ester linkage between the fatty acids (Cermak and Isbell, 2004). Figure 1 illustrates the structure of (A) a TAG-estolide and (B) a fatty acid estolide. TAG-estolide is used as a generic term defining a triacylglycerol oil in which additional fatty acids are esterified secondary to the glycerol "backbone". More specific nomenclature is used where the total number of fatty acids is known, for example a pentaester-TAG has 5 fatty acids associated with a single glycerol "backbone".

Natural TAG-estolides have been detected in the seed oil of a number of plant species including Kamala (*Mallotus philippinensis*, Rajiah et al., 1976) and the tallow tree (*Sapium sebiferum*, Sprecher et al., 1965) and are associated with the presence of fatty acids that contain a hydroxyl group (HFAs). A detailed analysis of the occurrence and structure of these lipids has been reported for the genus *Lesquerella* (Hayes et al., 1995). In a survey of 25 *Lesquerella* species the predominant estolide moiety was observed to be a monoestolide composed of a single HFA esterified to a second HFA attached to glycerol. The TAG-estolides were characterized by the presence of free hydroxyl groups. In the fungal kingdom, TAG-estolides account for almost all of the neutral lipids in the sclerotia of the ergot fungus (*Claviceps purpurea*), a pathogen of cereal crops and grasses (Morris and Hall, 1965; Batrakov and Tolkachev, 1997). Estolide structure differed from that reported from *Lesquerella*. Although ergot oil, extracted from mature sclerotia, contains the HFA ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid, 18:1-OH) at levels of up to 50% of the total fatty acids, IR spectroscopy has indicated that no free hydroxyl groups are present. Detailed analysis has shown that the predominant AG species are pentaester-TAG (5-AG, around 65%) and tetraester-TAG (4-AG around 27%), both containing monoestolides in which the secondary fatty acid does not contain a hydroxyl group (Batrakov and Tolachev, 1997).

A variety of techniques have been used to detect and quantify synthetic and natural estolides and TAG estolides. These include the use of thin layer and column chromatography, high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) to separate the acyl-species (Morris and Hall, 1965; Payne-Wahl, 1979; Hayes et al., 1995; Batrakov and Tolachev, 1997) and nuclear magnetic resonance (NMR), mass spectrometry, and Fourier transform infrared spectroscopy (FTIR-spectroscopy) to demonstrate the presence of the estolide linkages (Schulten et al., 1986; Erhan et al., 1996; Cermak and Isbell, 2003). Chemical and enzyme (lipase) mediated hydrolysis and gas chromatography has been applied to characterize the estolides moieties of TAG-estolides (Morris et al., 1965; Madrigal and Smith 1982; Batrakov and Tolkachev, 1997). Electrospray ionization tandem mass spectroscopy (ESI-MS/MS) has also been used to demonstrate the presence of AG-estolides as a minor component of castor oil (Lin et al., 2006).

The large scale utilization of synthetic estolides is currently limited by the cost of their production. The identification of natural sources of estolide may offer an opportunity to bring these novel lipids into more widespread use. As part of our ongoing research on natural sources of hydroxy fatty acids we were interested in developing techniques that could be used to detect the presence of estolides in intact biological material, or that would only require small amounts of oil. In this report we describe the application of ^{1}H-NMR to demonstrate the presence of estolides in intact seeds. This work was complimented by the development of a matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) method to provide further information on estolide structure using small amounts of extracted seed and fungal oil.

2. Experimental

Materials

Seeds of *Lesquerella fendleri*, and *Lesquerella lyrata* were kindly provided by Dr David Dierig (USDA ARS National Centre for Genetic Resources, Ft Collins, CO, USA). Mature sclerotia from *Claviceps purpurea* were collected locally from naturally infected Rye (*Secale cereale*). Castor oil (*Ricinus communis*) was purchased from Natural Sourcing LLC, CT, USA. Triacylglycerols standards and 2, 4, 6-trihydroxyacetophenone were purchased from Sigma-Aldrich Co. (St Louis, MO), α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Waters (Milford, MA).

Extraction of lipids and gas chromatography of fatty acid methyl esters.

Lipids were extracted from *Lesquerella* seeds or ergot sclerotia by grinding in hexane. After centrifugation for 3 minutes at 2000 g to remove particles, the hexane was removed to a clean tube, evaporated under a stream of nitrogen at room temperature and lipids were resuspended in a small volume of chloroform. For determination of

total fatty acid composition, oil samples, lightly crushed sclerotia or seeds were transmethylated by refluxing for 16 hours in sealed Pyrex tubes containing 2mL 1M HCl in methanol (Supelco, Bellefonte, PA, USA) and 500µL of hexane. After cooling, 2 mL of 0.9% NaCl was added and FAMES were recovered by collecting the hexane phase. Gas chromatography of FAMES was conducted using an Agilent 6890N GC fitted with a DB-23 capillary column (0.25 mm x 30 m, 0.25 µM thickness; J & W; Folsom, California, USA) and flame ionization detector, as described previously (Kunst et al., 1992).

^{1}H MAS-NMR analysis

For analysis of intact seed, spectra were acquired on an 8.46 T (360 MHz ^{1}H frequency) Bruker Avance NMR spectrometer using a 7 mm outer diameter double-resonance magic-angle spinning probe. Samples were spun at a rate of 3.0 kHz and, in cases where there was insufficient sample to fill the rotor, glass beads were used for balancing to allow stable spinning. A sweep width of 40 kHz, acquisition time of 102.5 ms, pulse delay of 2.0s, and optimized (p/2) pulse width of 6.0 ms were employed to collect a total of 128 acquisitions.

Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry.

Analysis of oil samples was carried out on an AB Sciex 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Carlsbad, CA, USA.) equipped with an Nd:YAG laser, which operated at a wavelength of 355 nm, with a 3 to 7 ns pulse width, and a pulse frequency of 200Hz. The mass spectrometer was operated in the positive ion reflectron mode, with a scanning range from m/z 800 to 4000. To ensure good mass accuracy, the default calibration in MS mode was updated with a standard mixture of six peptides which contained des-Arg1 bradykinin (m/z 904.468), angiotensin I (m/z 1296.685), Glu1 fibrinopeptide B (m/z 1570.677), and three ACTH clips (1-17, m/z 2093.087; 18-39, m/z 2465.199 and 7-38, m/z 3657.929). Peptides were deposited on a 384-well MALDI plate (0.5 µL, Opti-TOF insert, Applied Biosystems) precoated with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (0.5 µL), as described by Zhang et al., (2010). For oil analysis, samples were dissolved in chloroform, and 2, 4, 6-trihydroxyacetophenone (THAP) was used as matrix, which was made up to 10 mg/mL in water:methanol (1:9, volume ratio) saturated with sodium chloride. Subsequently, 0.5 µL of THAP matrix was spotted onto the MALDI plate followed by the same volume of sample solution. For MS/MS data collection, the instrument was operated in the positive ion 1kV MS/MS mode using air as the collision gas. Instrument default calibration was updated using the fragment ions from Glu1 Fibrinopeptide B. In both operation modes, 800 laser shots were collected and averaged for each spectrum.

3. Results and discussion

Fatty acid composition

The fatty acid compositions of castor oil, ergot oil and the seed oils of *L. fendleri* and *L. lyrata* as determined by GC-FAMES are given in table I. The major fatty acid in castor oil was ricinoleic acid accounting for over 88% of total fatty acids. The most abundant fatty acids in ergot oil were ricinoleic acid (29.6%), palmitic acid (hexadecanoic acid, 16:0, 27.2%), oleic acid (octadec-*cis*-9-enoic acid, 18:1, 17.9%) and linoleic acid (octadec-*cis*-9,12-dienoic acid, 18:2, 13.0%). HFAs were the major components of the oil from both *Lesquerella* species. In *L. fendleri*, the 20-carbon HFA lesquerolic acid (14-hydroxyeicos-*cis*-11-enoic acid, 20:1-OH) was most abundant at 56.5% with small amounts of auricolic (14-hydroxy-*cis*-11,17-dienoic acid 20:2-OH, 3.3%) and ricinoleic acid (0.6%) also present. Oil from *L. lyrata* contained 18-carbon HFAs at 41.1% densipolic acid (12-hydroxyoctadec-*cis*-9,15-dienoic acid, 18:2-OH) and 8.2% ricinoleic acid giving a total HFA content of nearly 50%. These results agreed with those previously published (Hayes et al., 1995; Batrakov and Tolkachev, 1997; Lin et al., 2002) but gave no indication whether TAG-estolides were components of the oils.

Oil separation by TLC

Separation of ergot oil and castor oil by thin layer chromatography (TLC) using a neutral lipid solvent system (Fig. 2) indicated that although both species contain ricinoleic as the predominant fatty acid, they clearly differ in structure. Castor oil showed the characteristic separation of TAG species with trihydroxy-TAG (3-OH TAG), dihydroxy TAG (2-OH TAG) and monohydroxy-TAG (1-OH TAG) separating according to their relative polarity conferred by the number of free hydroxyl groups in the individual lipid species. In contrast, the neutral lipids of the ergot oil chromatographed with an *Rf* value close to that of TAG containing no hydroxy fatty acids (Fig. 2, spot a), suggesting the absence of free hydroxyl groups. Lipid profiles of the two *Lesquerella* species were similar to each other, with the major difference being in the region with an *Rf* value close to that of 2OH-TAG from castor oil where a double spot was seen in the oil from *L. lyrata* (Fig. 2 spot b). Use of TLC to separate the lipids from Ergot and the *Lesquerella* species therefore suggests that unusual glycerolipid may be present, but does not allow interpretation of structure.

Detection of estolides by non-destructive ^{1}H MAS-NMR analysis

High resolution ^{1}H magic angle spinning NMR (^{1}H MAS-NMR) has previously been used for the qualitative and quantitative analysis of seed oil in intact oilseeds (Rutar, 1989; Wollenberg, 1991). When applied to intact ergot

sclerotia, a characteristic spectrum (Fig. 3A) was observed resembling that of an oilseed. The dehydrated state and high oil content of this fungal structure (30-40% oil, Batrachov and Tolkachev, 1997) enabled the collection of high quality data and allowed for detailed comparison to the spectra obtained from the *Lesquerella* seeds (Fig. 3, B and C). The chemical shift and resonance assignment for the ergot sample is given in Table 2 (Derived from Wollenberg, 1991). HFAs have distinct resonance that can be used to demonstrate their presence in a sample (Lie Ken Jie and Cheng, 1993). Diagnostic signals at 3.554 ppm and 3.561 ppm from the C14 and C12 protons of lesquerolic acid and densipolic/ricinoleic acid respectively were seen in spectra from *L.fendleri* and *L.lyrata,* indicating the presence of free hydroxyl groups in the oil. In contrast, no signal corresponding to the C12 proton of ricinoleic acid was observed in the ergot sample, suggesting that free hydroxyl groups were not present in the oil. Spectra from ergot and *L. lyrata* contained signals at 4.856 and 4.858ppm respectively, close to the characteristic chemical shift (4.87ppm) reported for fatty acid estolides generated from castor and *Lesquerella* oil by chemical synthesis (Isbell and Cermak, 2002). Both of these species were previously reported to contain TAG-estolides in their oil. No signal at this resonance was observed in the *L fendleri* seeds, a species that has been reported not to produce TAG-estolides (Hayes at al., 1995). Evidence of the presence of unsaturated hydroxy fatty acids in all three samples was given by the resonance at 5.428 to 5.433ppm, assigned to the C10 protons of ricinoleic/densipolic acid and C12 protons of lesquerolic acid. ^{1}H MAS-NMR of intact seeds therefore appears to be a useful, non-destructive technique to demonstrate the presence of estolides in natural material. However this technique does not provide information on estolide structure.

Detection of estolides by MALDI-TOF-MS and MALDI-TOF MS/MS

Use of MALDI-TOF-MS to characterize TAG species in plant and animal oils is now a well established technique (Ayorinde et al., 1999; Lay et al., 2006; Picariello, 2007; Fuchs et al., 2010) and enables the rapid analysis of small samples without the requirement for upstream separation procedures such as liquid chromatography (LC). As the technique generates singly charged ions primarily, the ion mass to charge ratio (m/z) corresponds to the monoisotopic mass of a molecule, allowing accurate mass analysis of TAG species. Coupled with tandem mass spectrometry (MS/MS)(Cheng et al., 1998), this technique also enables structural information to be obtained from the target ions. For a detailed analysis of the oils from castor, ergot, and the *Lesquerella* species we conducted MALDI-TOF-MS and MALDI-TOF MS/MS using a THAP matrix made up with sodium chloride saturated solvent, to ensure that sodiated ($[M+Na]^{+}$) ions were the predominant species generated during the laser desorption. As no internal standards were used, the analysis was considered to be qualitative only.

MALDI-TOF-MS spectra are compared in Fig. 4. For castor oil, the majority of the molecular ions were grouped in the range 913.8 m/z to 973.8 m/z. The TAG species present in castor oil have been characterized in detail (Lin et al., 2002) and the major species, ordered by relative abundance, are RRR>RRL>RRO>RRLn>RRS>RRP. The most intense peaks observed in the castor MALDI-TOF-MS spectrum (Fig. 4A) therefore represent the m/z of the sodiated TAG species $[M+Na]^+$ present in the oil, with the highest intensity peak (955.5 m/z) corresponding to tri-ricinoleoyl-TAG, $[RRR+Na]^+$. To confirm the identity of these ions, further structural information was obtained by tandem mass spectrometry (MALDI-TOF MS/MS). Fig. 5 shows the spectrum of a representative ion $[RRL+Na]^+$ from the castor oil sample compared to that obtained from a known TAG standard, $[POP+Na]^+$, which contains only common fatty acids. The MS/MS analysis of the standard TAG ($[POP+Na]^+$ = 855.7 m/z) gave a simple spectrum with 4 major fragment ions (Fig. 5A). Two ions corresponding to the loss of palmitic acid and oleic acid respectively, $[M+Na-\underline{P}COOH]^+$ at m/z 599.5 and $[M+Na-\underline{O}COOH]^+$ at m/z 573.5 were observed, as were ions corresponding to the neutral loss of the sodium salts of these fatty acids, $[M+Na-(\underline{P}COONa)]^+$ at m/z 577.5 and $[M+Na-(\underline{O}COONa)]^+$ at m/z 551.5. A similar fragmentation pattern showing ions corresponding to the loss of a single acyl group, either as a fatty acid (FA) or a sodium salt of a fatty acid was observed with the stereospecific TAGs OOP and POL (data not shown). The highest intensity ions resulting from the fragmentation of $[RRL+Na]^+$ in the castor sample (Fig. 5B) corresponded to the loss of a fatty acid $(M+Na-\underline{FA}COOH]^+$, with the loss of fatty acid sodium salt $[M+Na-\underline{FA}COONa]^+$ ions being of very low relative intensity. Although the castor TAG species under analysis contained both HFA and common fatty acids, only the HFAs were detected as $[FA+Na]^+$ ions in the MS/MS spectra. Diagnostic ions of use in determining the fatty acid composition of the TAG species containing HFAs were therefore the loss of fatty acid $[M+Na-\underline{FA}COOH]^+$ and the presence of the sodiated HFA ion $[\underline{HFA}COOH+Na]^+$. Spectra were complicated by the occurrence of multiple isobaric TAG species in the oil samples (for example ROLn and RLL). For this reason we did not attempt to determine additional structural information. By tandem mass spectrometry analysis we were able to identify the 971.8 m/z ion detected in the castor oil sample as a TAG species containing dihydroxy-18:1 ($[Di\text{-}OH\text{-}18:1 +Na]^+$ = 337.3 m/z), a minor fatty acid in castor oil previously reported by Lin and coworkers (2009) (data not shown).

For ergot oil, the molecular ions observed in the MALDI-TOF-MS spectrum (Fig. 4B) were present as three major groups, corresponding to the 4-AG (m/z 1110.0 to 1188.0), 5-AG (1390.3 m/z to 1468.3 m/z) and 6-AG (1672.5 m/z to 1724.6 m/z) TAG-estolide species, as previously reported (Batrachov and Tolkachev, 1997) using a lipase digestion method. Oil from *L. fendleri* gave a simple spectrum with components corresponding only to TAGs, predominantly containing one or two lesquerolic acid moeties (data not shown). We did not detect any significant ions corresponding to TAG estolides or to tri-lesquerolic-TAG (LqLqLq) in *Lesquerella fendleri*. In contrast, the oil from *L. lyrata* species was dominated by 4-AG (1169.9 m/z to 1227.9 m/z) and 5-AG (1465.9 m/z to 1508.1 m/z) species (figure 4C), thus confirming the previous report of the presence of TAG-estolides in this species (Hayes et al., 1995).

The MALDI-TOF-MS analysis clearly demonstrated the presence of molecules in the ergot and *L. lyrata* oil samples with deduced molecular weights corresponding to TAG-estolides. To determine the composition of the estolides, further analysis was carried out using MALDI-TOF MS/MS. Spectra for the majority of the ions indicated that they represented a mixture of TAG species. Ions diagnostic for the presence of HFAs and estolides were, however, easily detected in both the ergot and *L. lyrata* oil samples. For the ergot oil, high intensity signals in the MS/MS spectra corresponded to loss of fatty acid $[M+Na-\underline{FA}COOH]^+$, loss of estolide $[M+Na-\underline{Estolide}COOH]^+$, and the sodium adduct of the estolide itself $[EstolideCOOH+Na]^+$. No $[HFA+Na]^+$ ions were observed in any spectra examined, suggesting that all HFAs were involved in estolide linkage to other fatty acids, as observed in the ^{1}H-NMR and TLC studies (above) and reported previously (Batrachov and Tolkachev, 1997) Representative MS/MS spectra of a 4-AG and 6-AG TAG-estolides from ergot are shown in Fig. 6. Two major fatty acid estolides were observed, a ricinoleic-palmitic estolide ($[\underline{PR}COOH+Na]^+$ = 559.4 m/z) and a Ricinoleic-Oleic estolide ($[\underline{OR}COOH+Na]^+$ = 585.4 m/z); structures are illustrated in Fig. 1. Estolides containing 3 fatty acids were also detected such as the ricinoleic-ricinoleic-palmitic estolide ($[\underline{PRR}COOH+Na]^+$ = m/z 839.6, Fig. 6B). Previous enzymatic characterization of 6-AG species from ergot has indicated that the predominant molecules had fatty acid estolides occupying all three positions of the glycerol backbone (Batrachov and Tolkachev, 1997). The presence of high intensity $[M+Na-\underline{FA}COOH]^+$ ions in MS/MS spectra derived from 6-AG species therefore suggests that the loss of fatty acid can result from the breaking of an estolide bond.

MALDI-TOF MS/MS spectra obtained for ions from the *L. lyrata* sample showed a similar pattern to the ergot sample with $[M+Na-\underline{FA}COOH]^+$, $[M+Na-\underline{Estolide}COOH]^+$ and $[EstolideCOOH+Na]^+$ being the high intensity ions. In the majority of the TAG-estolide species analysed $[HFA+Na]^+$ ions were also observed, confirming the presence of HFAs in the molecule containing hydroxyl groups not involved in an estolide linkage. Representative MS/MS spectra of *L. lyrata* are shown in Fig. 7. In comparison to the ergot oil, the estolides of *L. lyrata* consisted only of two ester linked HFAs, either a Densipolic-densipolic estolide, or a densipolic+ricinoleic estolide, with the fatty acid order of the latter being uncertain. These results are consistent with the previous report in which the estolide composition of oil was examined using supercritical fluid chromatography and lipase-catalysed hydrolysis (Hayes et al., 1995).

MALDI-TOF-MS and MS/MS using small amounts of oil from the species of interest is a highly informative method for the detection and characterization of TAG-estolides. The ions observed in the MS/MS spectra in this study are similar to those reported from previous work characterizing a low abundance TAG-estolide from castor oil by LC-ESI-MS/MS (Lin et al., 2006). MALDI-TOF MS has the advantage of high throughput, short analysis time, tolerance of contaminants such as salts or detergents and allows for the re-analysis of samples from the MALDI plate as needed, all without prior separation of the oil components. The technique does have limitations for the analysis of isobaric TAG species, and in addition, due to instrument hardware limitations, when isolating an ion for MS/MS analysis, the m/z range being isolated is ~6-8 m/z units meaning that some interference from other compounds having similar mass can occur. The approach reported here is qualitative and it is likely that the

increased polarity of TAGs containing HFA may give rise to higher signal intensity than seen with TAGs containing common fatty acids. Nevertheless, despite these limitations, high throughput screening of many different plant species will be a valuable tool for the assessment of the abundance and distribution of natural TAG-estolides.

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Table 1 **Fatty acid composition**

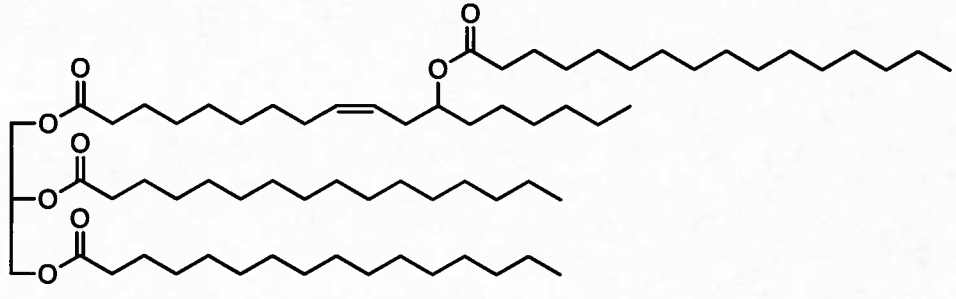
Fatty acid (abbreviation used in figures)	*C. purpurea* sclerotia (%)	Castor oil (%)	*L. fendleri* (%)	*L. lyrata* (%)
Palmitic acid, 16:0 (P)	27.2	1.3	1.1	5.7
Palmitoleic acid, 16:1$^{\Delta 9}$ (Po)	3.3	T	0.5	1.4
Stearic acid, 18:0 (S)	6.2	0.7	1.9	4.6
Oleic acid, 18:1$^{\Delta 9}$ (O)	17.9	2.0	13.8	13.7
Cis-vaccenic acid, 18:1$^{\Delta 11}$ (V)	1.3	0.4	1.6	4.3
Linoleic acid, 18:2$^{\Delta 9,12}$ (L)	13.0	6.4	7.0	2.3
Linolenic acid, 18:3$^{\Delta 9,12,15}$ (Ln)	-	-	11.3	12.1
Arachidic acid, 20:0 (A)	-	T	0.2	0.7
Eicosenoic acid, 20:1$^{\Delta 11}$ (E)	-	-	0.9	0.2
12-hydroxy palmitoleic acid, 16:1-OH (HPo)	-	-	-	2.1
Ricinoleic acid, 18:1-OH (R)	29.6	88.1	0.6	8.2
Densipolic acid, 18:2-OH (D)	-	-	-	41.1
Lesquerolic acid, 20:1-OH (Lq)	-	-	56.5	-
Auricolic acid, 20:2-OH (Au)	-	-	3.3	-
Total Hydroxy fatty acids (HFA)	**29.6**	**88.1**	**60.4**	**51.4**

T Present, but level too low to accurately quantify.

Table 2. ^{1}H MAS-NMR of TAG-estolides: Assignment of chemical shift

Chemical shift (ppm)	Assignment
0.895	-CH_2-C***H***$_3$
0.944 *L. lyrata*	-CH=CH-CH_2-C***H***$_3$
1.294	-(C***H***$_2$)$_n$-
1.588	-O-CO-CH_2-C***H***$_2$-
2.036	-C***H***$_2$-CH=CH-
2.187 *L. fendleri*	-CH=CH-C***H***$_2$-CHOH-CH_2-
2.248	-O-CO-C***H***$_2$-
2.767	-CH=CH-C***H***$_2$-CH=CH-
4.090 and 4.296	***H***$_2$CO- (α-C of glycerol)
4.856	-***H***COR- (estolide signal)
5.222	-***H***CO- (β-C of glycerol)
5.326	-C***H***=C***H***-
5.428	-CH=C***H***-CH_2-CHOH-
3.554 *L. fendleri*	-C***H***OH- (HFA signal)

A



B

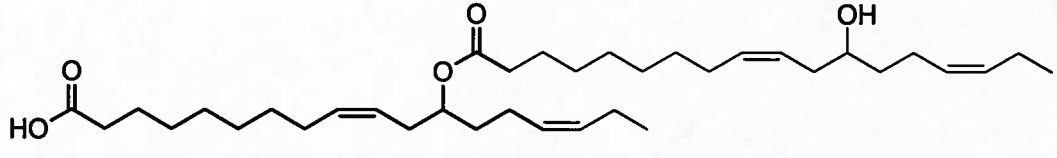


Figure 1

Figure 2

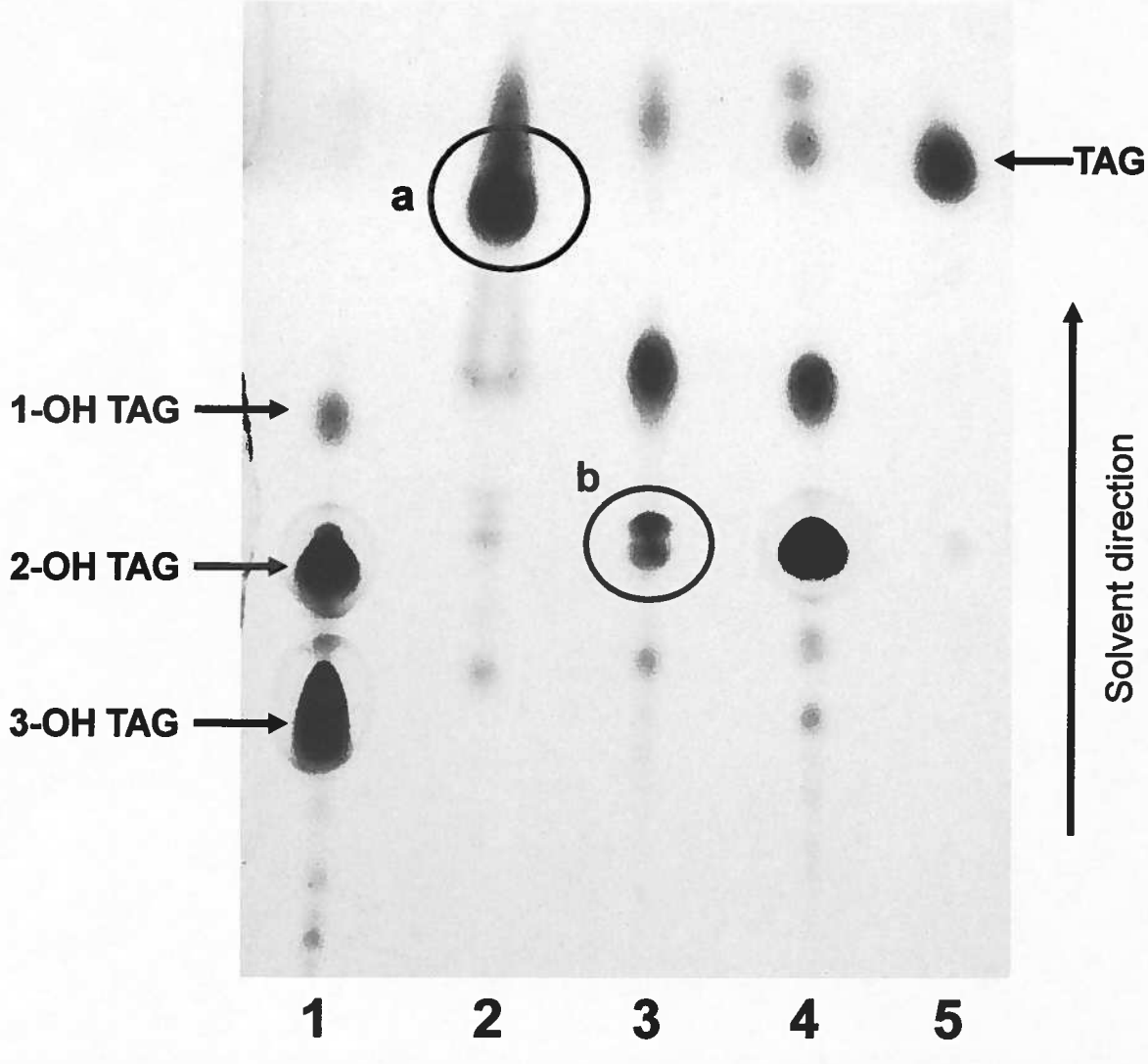


Figure 3

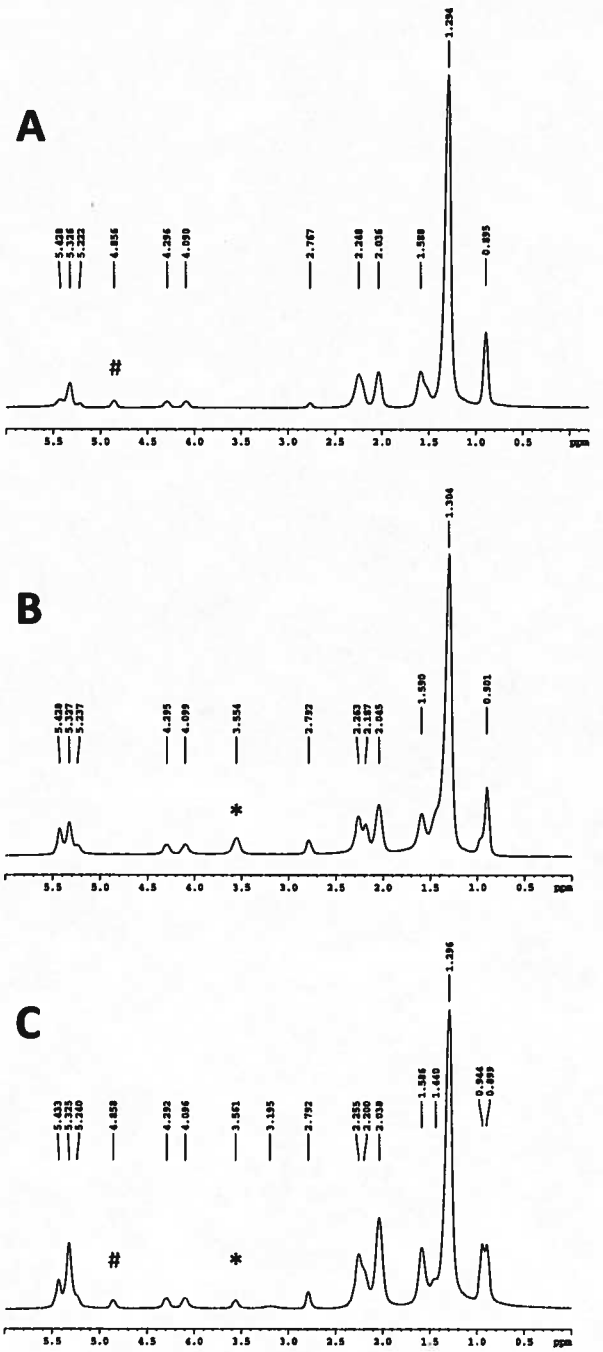
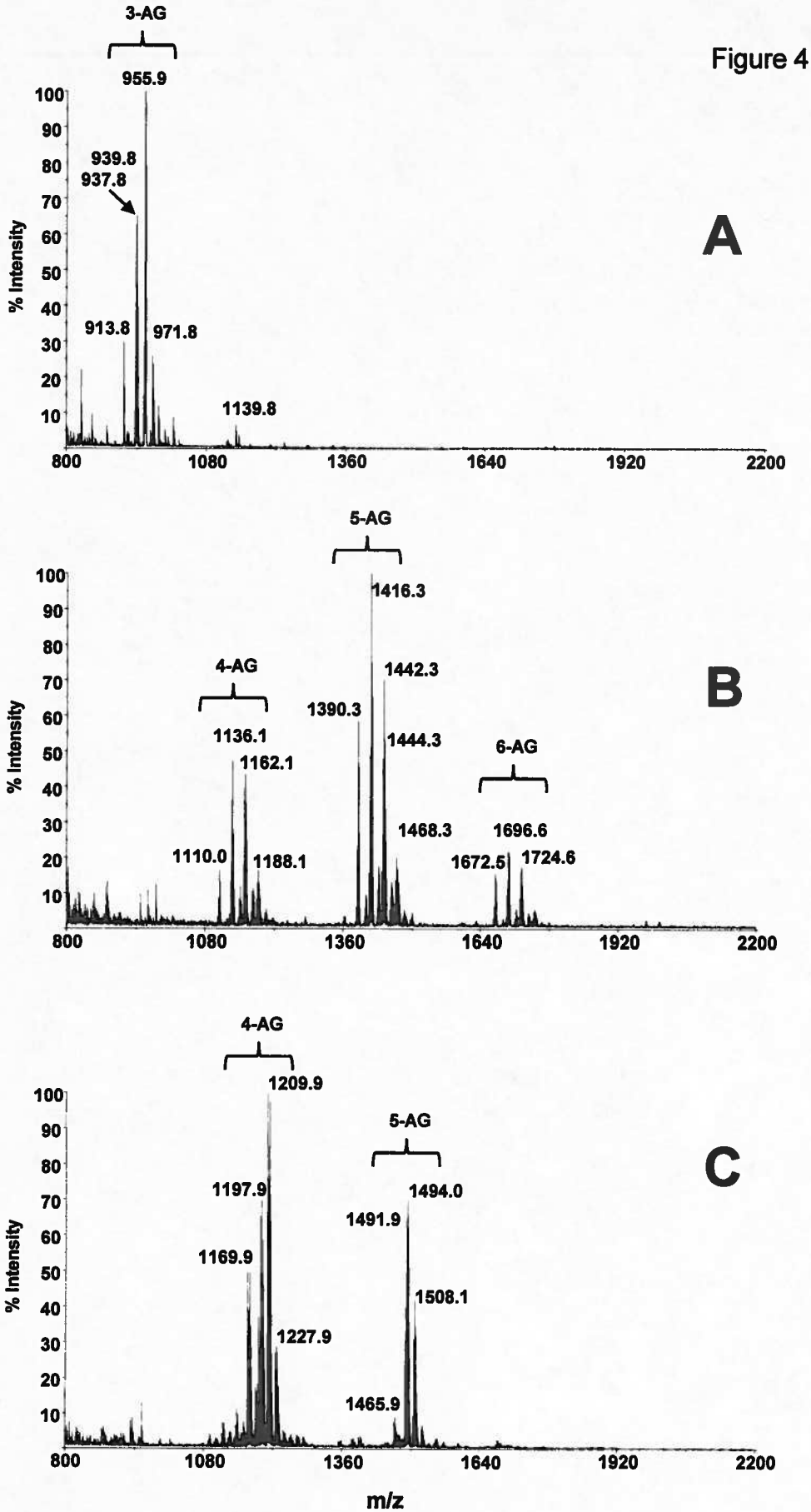
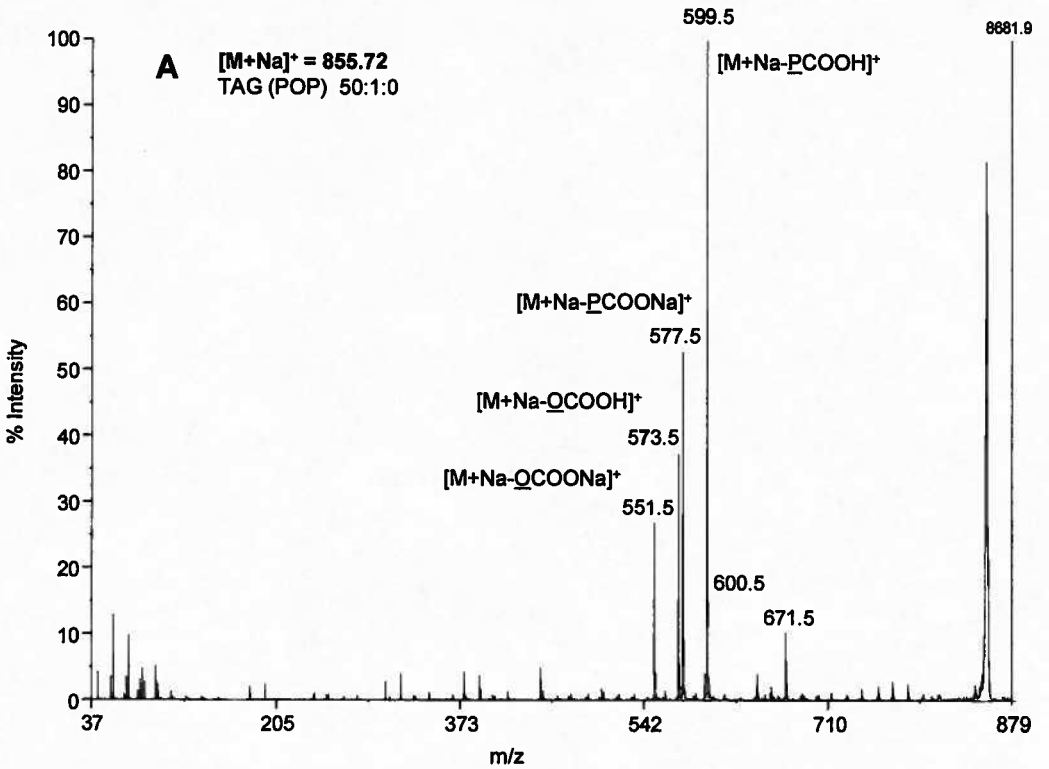


Figure 4


3-AG
955.9
939.8
937.8
913.8
971.8
1139.8
A
% Intensity
100
90
80
70
60
50
40
30
20
10
800
1080
1360
1640
1920
2200
5-AG
1416.3
4-AG
1442.3
1390.3
1136.1
1444.3
1162.1
6-AG
1468.3
1696.6
1110.0
1188.1
1672.5
1724.6
B
4-AG
1209.9
5-AG
1197.9
1494.0
1491.9
1169.9
1508.1
1227.9
1465.9
C
m/z


A
[M+Na]+ = 855.72
TAG (POP) 50:1:0
599.5
[M+Na-PCOOH]+
8681.9
[M+Na-PCOONa]+
577.5
[M+Na-OCOOH]+
573.5
[M+Na-OCOONa]+
551.5
600.5
671.5
% Intensity
m/z
100
90
80
70
60
50
40
30
20
10
0
37
205
373
542
710
879

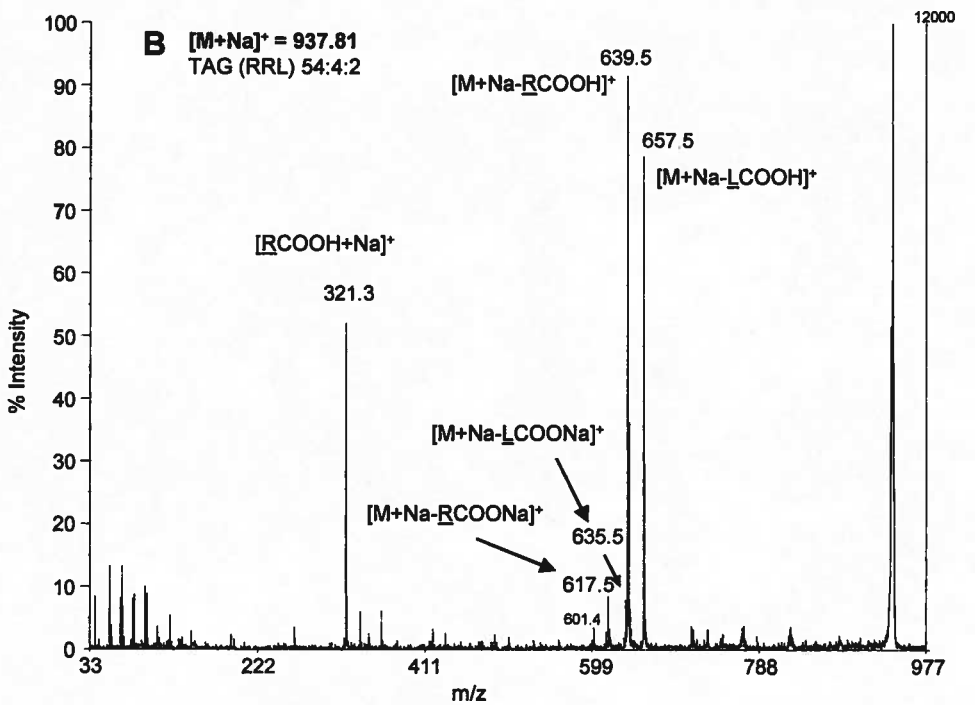

B
[M+Na]+ = 937.81
TAG (RRL) 54:4:2
639.5
[M+Na-RCOOH]+
12000
657.5
[M+Na-LCOOH]+
[RCOOH+Na]+
321.3
[M+Na-LCOONa]+
[M+Na-RCOONa]+
635.5
617.5
601.4
% Intensity
m/z
100
90
80
70
60
50
40
30
20
10
0
33
222
411
599
788
977

Figure 5

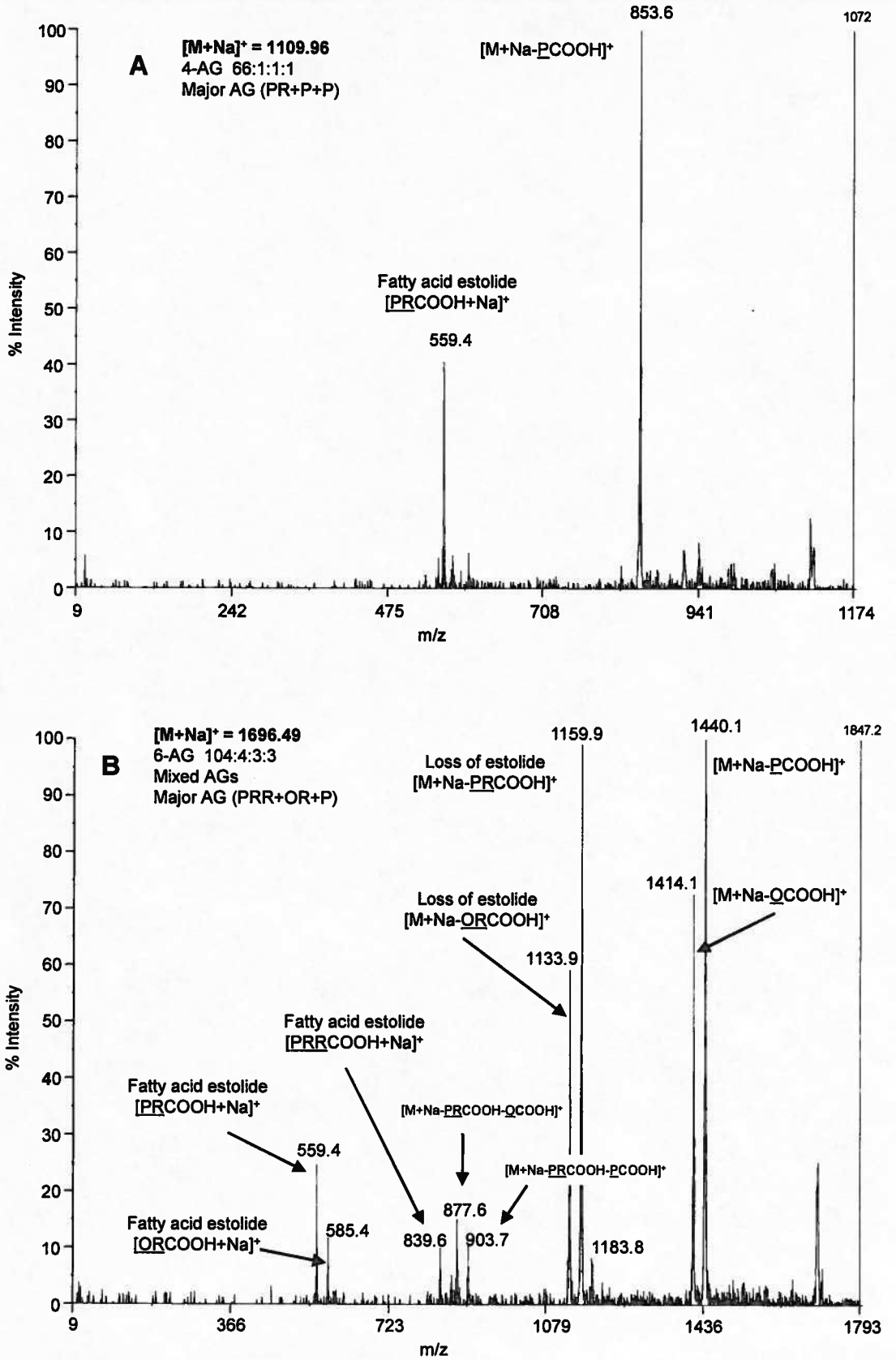
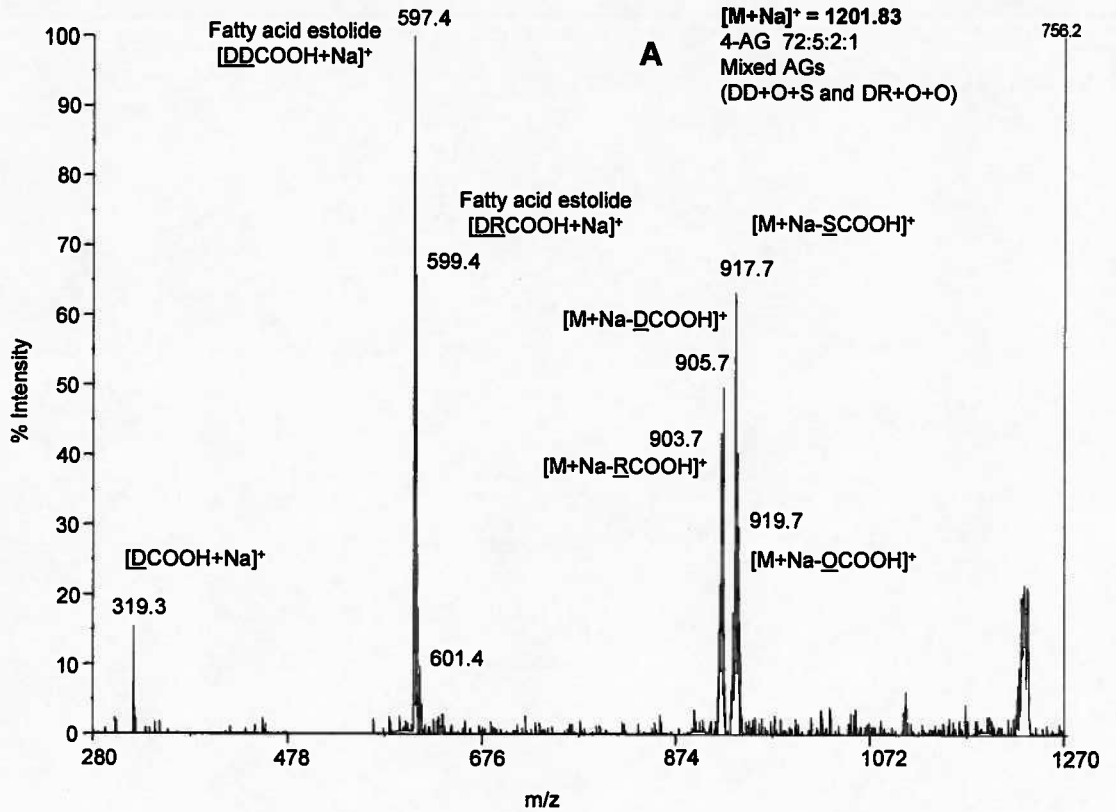

A
[M+Na]+ = 1109.96
4-AG 66:1:1:1
Major AG (PR+P+P)
[M+Na-PCOOH]+
853.6
1072
Fatty acid estolide
[PRCOOH+Na]+
559.4
% Intensity
m/z
B
[M+Na]+ = 1696.49
6-AG 104:4:3:3
Mixed AGs
Major AG (PRR+OR+P)
Loss of estolide
[M+Na-PRCOOH]+
1159.9
1440.1
1847.2
[M+Na-PCOOH]+
1414.1
[M+Na-OCOOH]+
Loss of estolide
[M+Na-ORCOOH]+
1133.9
Fatty acid estolide
[PRRCOOH+Na]+
Fatty acid estolide
[PRCOOH+Na]+
[M+Na-PRCOOH-QCOOH]+
559.4
[M+Na-PRCOOH-PCOOH]+
877.6
Fatty acid estolide
[ORCOOH+Na]+
585.4
839.6
903.7
1183.8
% Intensity
m/z

Figure 6


A
[M+Na]+ = 1201.83
4-AG 72:5:2:1
Mixed AGs
(DD+O+S and DR+O+O)
Fatty acid estolide
[DDCOOH+Na]+
597.4
Fatty acid estolide
[DRCOOH+Na]+
599.4
[M+Na-SCOOH]+
917.7
[M+Na-DCOOH]+
905.7
903.7
[M+Na-RCOOH]+
919.7
[M+Na-OCOOH]+
[DCOOH+Na]+
319.3
601.4
756.2
% Intensity
m/z
100
90
80
70
60
50
40
30
20
10
0
280
478
676
874
1072
1270

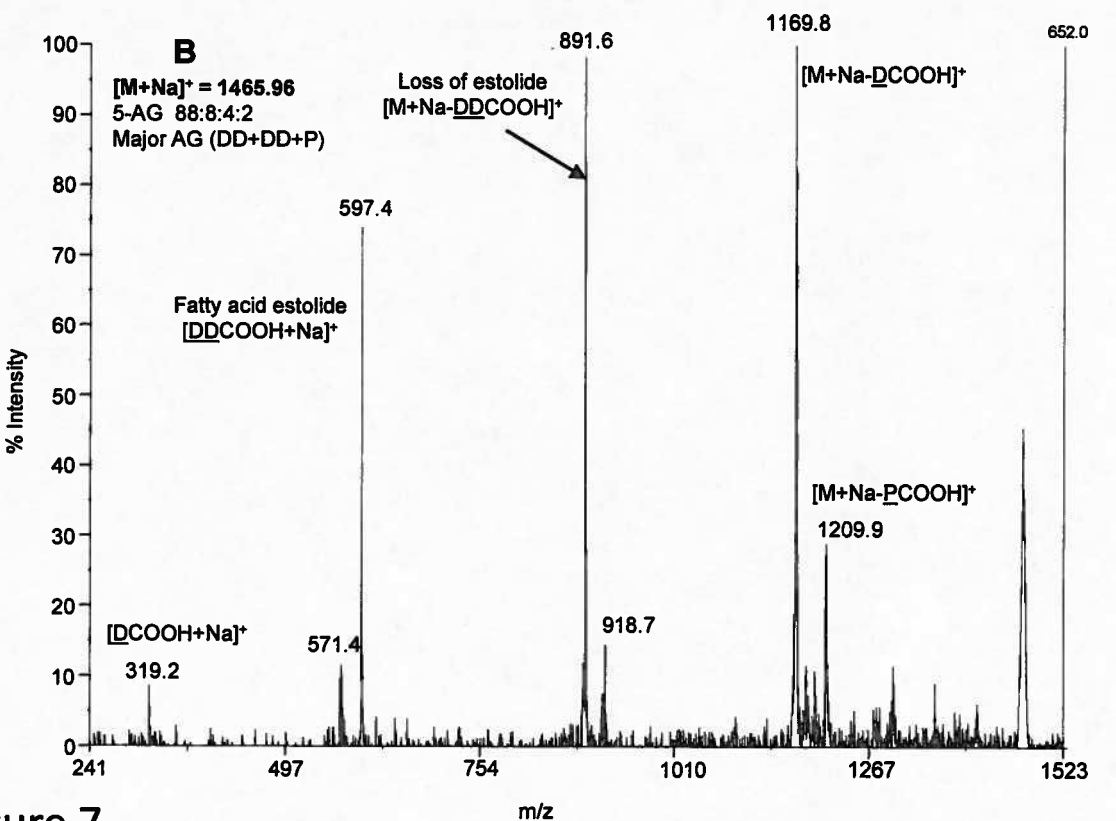

B
[M+Na]+ = 1465.96
5-AG 88:8:4:2
Major AG (DD+DD+P)
Loss of estolide
[M+Na-DDCOOH]+
891.6
1169.8
[M+Na-DCOOH]+
652.0
597.4
Fatty acid estolide
[DDCOOH+Na]+
[M+Na-PCOOH]+
1209.9
[DCOOH+Na]+
319.2
571.4
918.7
% Intensity
m/z
100
90
80
70
60
50
40
30
20
10
0
241
497
754
1010
1267
1523

Figure 7

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Figure legends.

Figure 1.
Cartoon showing structure of TAG and fatty acid estolides.
A: A representation of a TAG-estolide. B: Monoestolide of densipolic acid (DD-estolide).

Figure 2.
Separation of lipids by Thin Layer Chromatography.
Lipids were separated on silica gel 60 coated plates using a neutral lipid solvent system (Hexane:diethyl-ether:acetic acid, 90:110:3 by volume). *1* – castor oil, *2* – ergot oil, *3* – *Lesquerella lyrata* oil, *4* – *Lesquerella fendleri* oil, *5* – TAG standard (Tri-oleate, OOO).

Figure 3.
^{1}H MAS-NMR spectra.
A: Intact sclerotia of the ergot fungus *Claviceps purpurea*, B: Whole seeds of *Lesquerella fendleri*, C: Whole seeds of *Lesquerella lyrata*. * indicates resonance characteristic of a hydroxyl group, # indicates resonance diagnostic for fatty acid and TAG estolides.

Figure 4.
MALDI-TOF-MS spectra of oil samples.
A: Castor oil, B: Ergot oil, C: *Lesquerella lyrata* oil. For each group of signals, only the peaks with highest intensity are labeled.

Figure 5.
MALDI-TOF-MS/MS spectra of TAGs.
A: Stereospecific TAG standard (POP, precursor ion 855.73 m/z), B: RRL-TAG, precursor ion 937.81 m/z, from castor oil. Predominant TAG species and diagnostic fragment ions are indicated. Acyl-glycerol nomenclature A:B:C where A = number of carbons in fatty acid chains, B = number of double bonds, C = number of hydroxyl groups.

Figure 6.
MALDI-TOF-MS/MS spectra of representative TAG-estolides from ergot oil.
Predominant molecular species are indicated. Acyl-glycerol nomenclature A:B:C:D where A = number of carbons in fatty acid chains, B = number of double bonds, C = number of hydroxyl groups (free and estolide), D = number of fatty acid to fatty acid ester bonds (estolide linkage). Highest intensity ions are labeled and diagnostic fragment ions are indicated.

Figure 7.
MALDI-TOF-MS/MS spectra of representative TAG-estolides from Lesquerella lyrata oil.
Predominant molecular species are indicated.