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*Highlights

- speciation of selenium compounds in plant oil
- detection and characterization of selenotriglycerides
- combined HPLC-ICP MS/ESI Orbitrap MS³ analysis

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Speciation of selenium in selenium-enriched sunflower oil by HPLC-ICP MS/ electrospray - Orbitrap MS/MS

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ABSTRACT

The reaction of sunflower oil with selenite produces a complex mixture of selenitriglycerides with anti-oxidant and anticancer properties. In order to get an insight into the identity and characteristics of the species formed an analytical approach based on the combination of HPLC with ⁷⁸Se specific selenium detection by inductively coupled plasma mass spectrometry (ICP MS) and high resolution (100,000) high mass accuracy (<1 ppm) molecule specific detection by electrospray Orbitrap – MS³ was developed. Two types of samples were analysed: sunflower oil dissolved in isopropanol and methanol extract of the oil containing 65% of selenium. HPLC – ICP MS showed 14 peaks, 11 of which could also be detected in the methanol extract. Isotopic patterns corresponding to molecules with one or two selenium atoms could be attributed by Orbitrap MS at the retention times corresponding to the HPLC-ICP MS peak apexes. Structural data for these species was acquired by MS² and MS³ fragmentation of protonated or sodiated ions using high energy collisional dissociation (HCD). Eleven selenium-containing triglycerol derivatives resulting from the oxidation of one or two double bonds of the linoleic acid and analogous derivatives of glycerol mixed linoleate(s)/oleinate(s) have been identified for the first time. The presence of these species was confirmed by the targeted analysis in the total oil isopropanol solution. Their identification corroborated the predicted elution order in reversed-phase chromatography: LLL, LLO, LOO, OOO of which the extrapolation allowed the prediction of the identity (OOS, OSS) of the nonpolar species detected by ICP MS in the oil but not detected by electrospray MS.

Keywords: selenium, speciation, plant oil, Orbitrap, HPLC-ICP MS, fatty acid

INTRODUCTION

Selenium is an essential micronutrient that plays a vital role in most of the biochemical and physiological processes including immune function, thyroid hormone metabolism and antioxidant defense systems[1][2]. The deficiency of selenium is a global problem which is addressed by supplementation [3]. The superior availability of the organic selenium in comparison to its inorganic forms is well documented in the literature [4].

A mixture of selenitetrigerides (Selol) produced by the reaction of selenite with sunflower oil has recently been proposed as a novel non-toxic, highly bioavailable and active antioxidant[5] and antineoplastic agent.[6, 7] The speciation of selenium, which is the key to the understanding of the properties of Selol and the mechanisms of its reactivity, is largely unknown and expected to vary depending on the selenium-to-oil ratio used for the reaction [8]. Sunflower oil is a mixture of lipids composed mainly (approximately 96%) from triacylglycerols consisting of a glycerol backbone and different fatty acid moieties; the latter include principally linoleic acid (L, polyunsaturated omega-6, 59%), oleic acid (O, monounsaturated omega-9, 30%), stearic (S, saturated, 6%) and palmitic acid (P, saturated, 5%) [9]. The data available in the literature give variable ranges of different residues including LLL species between 30 and 43% [10-12], LLO 20-30% [10, 11], OOL 12-17% [10] and PLL 10-13.6 % [10, 11]. The combination of a long alkyl chain with a hydrophilic selenide moiety as a redox center is likely to confer glutathione-peroxidase (GPx)-like antioxidant activity as recently shown in a model study for fatty acid conjugates of water-soluble (±)-trans-selenolane-3,4-diol [13].

The complementary use of ICP MS and electrospray MS/MS detection in speciation studies [14, 15] has offered an unrivalled approach to the identification of the chemical forms of trace elements in biological systems. Most of the data have been obtained for water soluble compounds that can be

1 readily separated by different types of chromatography with mobile phases compatible with ICP MS
2 or electrospray detection. Examples of speciation analysis of water-insoluble species have been much
3 scarcer and mostly arsenolipids [16-21] and petroleum products [22].
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5 To date, there are no data on selenium speciation in oils. In addition to the insolubility in water, the
6 large number and diversity selenitetriglyceride derivatives make their separation and identification
7 difficult. The objective of this work was to optimize the separation of selenium species in oil and its
8 polar fraction using selenium-specific ICP MS detection with a focus on background correction and
9 quantitative elution in order to facilitate data mining in electrospray MS. The subsequent accurate
10 mass measurement and higher energy collision dissociation (HCD) fragmentation MS² and MS³
11 producing a large population of fragment ions were expected to allow the identification and an
12 insight into structural characterization of the selenium species detected by ICP MS.
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18 **MATERIALS AND METHODS**

19 ***Reagents***

20 LC-MS solvents acetonitrile (ACN), isopropanol (IPA), acetone, methanol, hexane, cyclohexane, acetic
21 acid, HNO₃ (69–70%) and H₂O₂ (30%) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier,
22 France). Mili-Q water (18 MΩ cm) was used throughout.
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27 ***Samples***

28 SelolTM (50 mg/ml Se (IV)) was synthesized by reaction of Se(IV) with sunflower oil at the Department
29 of Bioanalysis and Drug Analysis at the Medical University of Warsaw.[23]
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32 ***Instrumentation***

33 HPLC – ICP MS was performed with an Agilent series 1200 instrument equipped with an autosampler
34 and coupled to an Agilent 7700 ICP-MS (Agilent, Hachi-oji, Japan). For HPLC – MS/MS analysis a
35 Dionex system (Ultimate 3000) was coupled to an ESI-LTQ Orbitrap Velos (Thermo Scientific,
36 Waltham, MA, USA) operated in the positive ionization mode. The chromatographic column was, in
37 both cases, Zorbax eclipse XDB-C₁₈ (4,6 mm x 150 mm x 5 μm) column (Agilent Technologies).
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42 ***Procedures***

43 *Sample preparation.* The polar fraction of SelolTM was obtained by partitioning 600 μl of SelolTM (50
44 mg Se/ml) between hexane (1500 μl) and methanol-water (9:1). A volume of 900 μl of the MeOH
45 fraction was recovered for analysis. For the analysis of the entire sample, an aliquot of SelolTM was
46 dissolved in isopropanol to reach a concentration of at 5 ppm Se. The methanol extract (20μl) was
47 dissolved in the mobile phase (980 μl) (IPA: ACN) (62.5 : 37.5 v/v).
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51 *Determination of the recoveries.* The extraction and HPLC recoveries were determined by comparison
52 of the Se signal intensity in the extract and total collected HPLC eluent with the solution of Selol in
53 isopropanol. The samples were diluted 20 times isopropanol in order to minimize the matrix effect.
54

55 *HPLC – ICP MS/ESI MS.* The HPLC and ICP MS conditions are given in **Table 1**. For MS analysis full
56 enhanced MS scans (resolution 100,000) were acquired in the positive ion mode, over the m/z range
57 200-2000. The electrospray voltage was set to 4kV, the capillary temperature 350 °C and the S-lens
58 RF level 68%. The auxiliary and sheath gas flows (both nitrogen) were optimized 20-75 (arbitrary
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units). The optimization aimed at the minimizing of the fragmentation in the source [24]. HCD activation of ions was applied in MS² and MS³ experiments, with 35 normalized collision energy and 0.1 ms activation time. The analyses were carried out using the same settings for all analytes.

RESULTS AND DISCUSSION

Solubility of SelolTM and partitioning of selenium species

SelolTM is an oily liquid. In order to be chromatographed, it had to be dissolved in an appropriate solvent. A number of solvents including: acetone, dichloromethane, dimethyl sulfoxide, dimethylformamide, acetonitrile, water, isopropanol, methanol, hexane, cyclohexane, acetic acid and their mixtures: methanol-water (9:1), methanol-hexane (1:1), methanol-dichloromethane (1:2), methanol-isopropanol, acetonitrile-isopropanol (7:3) were tested. The total solubility (no visible residue) was observed using isopropanol, acetic acid, acetone, dichloromethane, hexane, cyclohexane, methanol-dichloromethane (1:2), isopropanol-acetonitrile (2:1). Isopropanol was chosen as the solvent.

The polar selenium species were recovered from the mixture using a modified extraction method of Francesconi *et al.*[25] for arsenolipids in fish oil consisting in partitioning the species between hexane and 90 % (v/v) methanol. The fraction of selenium recovered in the methanol was $64.0 \pm 0.4\%$.

Chromatographic separation of selenium species

Because of the complexity of the investigated sample, a chromatographic separation step was required prior to its ESI MS characterization. The HPLC method was optimized using ICP MS Se-specific detection in order to assure the best possible separation of the selenized analytes and allow the determination of the chromatographic recovery. Two separation modes: reversed phase (Zorbax Eclipse xdb-C18 (4,6 x 150 mm x 5 μ m)) and HILIC (TSKgel Amide-80 (1 x 250 mm x 5 μ m)), were tested for the total (isopropanol solution) and polar (methanol extract) fractions of SelolTM. Isopropanol-acetonitrile (able to dissolve Selol completely) was chosen as the mobile phase and the elution gradient was optimized. The chromatogram of the total SelolTM is shown in **Fig. 1a** (bold line). The steep increase in the baseline was supposed to be due to the increase in ICP MS sensitivity with the increasing concentration of isopropanol. This hypothesis was confirmed by the analysis of a selenium standard added at the same concentration to the eluents A and B. An increase in the sensitivity with the increase in the concentration of ACN is demonstrated in **Fig. 1a** (thin line).

A chromatogram corrected for the change in intensity is shown in **Fig. 1b**. It shows about 10 peaks of the different intensities. At these conditions the recovery of selenium was $98 \pm 2\%$.

A chromatogram of the methanol extract acquired in the same conditions (**Fig. 1c**) shows 11 peaks of different intensities with retention times corresponding to those in the total SelolTM chromatogram. No peaks are observed after 22 min corresponding to 40% ACN ("a polarity cut-off"). Note that the fraction of selenium calculated from the intensity-corrected chromatogram (Fig. 1b, 0-22 min) corresponds to ca. $60 \pm 4\%$ of the total selenium which is in agreement with the methanol partition recovery.

HPLC - electrospray MS detection for the selenium-lipid species

The principle of the approach consisted in reproducing the chromatograms in **Fig. 1** (ac) using electrospray MS detection to assign an accurate (<1ppm) molecular mass to the peaks from the

1 selenium-containing compounds. The major problems in electrospray ionization of lipids include (i)
2 difficulty in the optimization of ionization conditions because of the high probability of in-source
3 fragmentation, and (ii) their affinity for sodium, potassium and ammonia cations resulting in multiple
4 molecular ions detection which makes the interpretation of spectra difficult even from a single class
5 of lipids,[26] and (3) contribution of solvents to the formation of adducts, which altogether results in
6 complex spectra.[27, 28] The to date reports did not concern selenium and did not use HR
7 spectrometry. It was therefore expected that the 100,000 and sub-ppm mass accuracy should largely
8 facilitate data interpretation. Preliminary optimization was carried out using a series of triglycerides
9 standards in order to minimize their in-source fragmentation. Pseudo-molecular ions could thus be
10 obtained.

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13 The analysis of the ESI MS data sets acquired in the chromatographic conditions as in Fig. 1
14 aimed at finding the isotopic patterns corresponding to the presence of one or more selenium atoms.
15 The success of this approach of the methanol extract is illustrated in **Fig. 2 a-c** (left panels). Peaks in
16 the extracted ion chromatograms of Se-containing ions were found to match the peaks in the ICP MS
17 chromatogram. The putative experimental formulae (given in Table 2) could be determined on the
18 basis of the experimental molecular masses. Any of the 11 species observed contained 57 carbon
19 atoms. Almost all the XIC chromatograms consisted of more than one peak; the major one eluting
20 first in the chromatogram. The presence of multiple peaks most probably results from the presence
21 of isomers corresponding to modifications of different fatty acid residues in LLL and OOO (position
22 *sn-1, 2 or 3*) species or different configurations of mixed LLO/LOL, OOL/OLO triglycerides. Similar XIC
23 morphology was shown elsewhere for sunflower oil demonstrating that a saturated (palmitic) acid
24 was typically located at positions *sn-1* or *sn-3* whereas unsaturated ones were mostly found at the
25 *sn-2* position [26].
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32 HPLC - MS² and MS³ analysis

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34 The identification of the fatty acids residues composition of triglycerides becomes a
35 challenge because of the difficulties to produce controlled sample fragmentation, the presence of
36 many closely related structures and, especially of structural isomers present identical
37 pseudomolecular ions and fragments [27, 28]. It was reported that, in MS/MS, the loss of the fatty
38 acid at position *sn-2* was least favored with preferential loss of residues at positions *sn-1/3* [26]. The
39 additional difficulty concerns locating the position of the double bond. All this makes the high
40 resolution and mass accuracy essential, and MSⁿ (n>2) of considerable interest. The optimization of
41 the working conditions was carried out using a series of triglycerides standards. A set of data on
42 fragments down to MS³ level were acquired facilitating the interpretation of spectra
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46 The chromatography was repeated in the same conditions for the targeted fragmentation
47 using high energy collisional dissociation (HCD) of the Se-containing ions with the option
48 fragmentation for the most intense ions (MS³ mode). An example of the fragmentation cascade for
49 species m/z 1013.6913 is given in **Fig. 3**. The fragmentation involved cleavage of individual fatty acid
50 residues, the phenomenon used by some authors to establish the contribution of different isomers in
51 mixed (eg., LLO/LOL or OOL/OLO) species [26]. The full list of the fragments is given in supplementary
52 information (Table S1). Note that, as expected on the basis of available literature,[26] most of
53 pseudomolecular ions and their fragments were detected as sodium adducts of the
54 selenitetriglycerides.
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58 Putative structures established on the basis of MS² and MS³ data are presented in the **Fig. 2**
59 **(right panel)**. The collected information allows the conclusion that the synthesis of selenite-
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1 triglycerides involves the breakage of the double bond(s) in the linoleic or oleic acid residue of the
2 glycerol followed by an addition of a hydrogen atom or an OH-group; the presence of two hydroxyl
3 groups at the neighboring C atoms allows the introduction of a Se=O group. The exact position of this
4 residue in the fatty acid chain could not be determined by MS; however, its position had to
5 correspond to that of the double bond in the substrate species, so either 9 and/or 12 or 9 in linoleic
6 and oleic acid, respectively. The species identified vary in terms of the presence of double bonds,
7 hydroxyl groups in addition to the $(-O)_2\text{-Se=O}$ group(s).

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9 The presence of three peaks in the XIC chromatograms (**Fig. 2**) seems to result from the Se
10 incorporation into different residues of the glycerol. According to this hypothesis, the most intense
11 peak would correspond to the selenization of the most sterically accessible fatty acid residue. Hence,
12 the proposed mechanism of the triglyceride selenization excludes the possibility of selenium
13 incorporation into species containing saturated fatty acid residues. Although the presence of mixed
14 (saturated/unsaturated) species could be expected, they were not detected.

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16 The species detected contained one (m/z 1063, 1031, 1047, 1029, 1013, 1033) or two (m/z
17 1111, 1113, 1115 and 1059) Se atoms (*cf.* Table 2). The exact structure of the Se-Se bond in the
18 species m/z 1111 could not be elucidated by MS; the possible hypothesis involves two Se(IV) atoms -
19 bound to each other by a double bond and to oxygen atoms (one by a double bond and the second
20 one by two single bonds creating oxygen bridges binding it to the fatty acid residue.

21 22 23 24 25 26 27 **Non-polar selenium species**

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29 The direct HPLC – electrospray MS of the SelolTM solution results in a very noisy background and is
30 relatively poor in information about the selenium species. The presence of the 11 species was
31 confirmed in the target analysis mode (MS^2 of the target molecular masses) but no other compound
32 could be detected. Nevertheless the HPLC – ICP MS chromatogram (**Fig. 2**) shows clearly the elution
33 of the 35% of selenium in the form of three intense peaks.

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36 An insight into the identity of these peaks can be gained by analysis of the structures and the elution
37 order of the identified species. This order is clearly influenced by the number of double bonds,
38 hydroxyl groups and selenium substitutions as shown in **Fig. 4**. The data acquired confirmed this
39 hypothesis for LLL, LLO, OOL, OOO. On this basis an elution order of $L_xO_yS_z$ (where $x+y+z = 3$) could be
40 established, as the hydrophobicity is known [10] to increase with the decreasing number of the
41 double bonds. It is therefore likely that the three prominent peaks beyond the “polarity cut-off”
42 correspond to OOS and OSS derivatives (including stearic acid present at ca. 6% in the oil). The
43 occurrence of palmitic acid residues can also be expected.

44 45 46 47 48 **Conclusions**

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50 The study allowed for the first time the characterization of a mixture of selenitetrigerides in oil
51 with putative anti-oxidant and anticancer properties. The development of HPLC-ICP MS using an
52 organic mobile phase made it possible to elute the entirety of the selenium and to detect 14 major
53 peaks. Electrospray MS enabled the identification of 11 of these compounds corresponding to the
54 glycerides with different fatty acid chains. The discussion of the elution order of the identified
55 compounds in the context of their physicochemical properties allowed the prediction of the identity
56 of the compounds detected by ICP MS but not identified by electrospray MS.

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Captions to Figures:

Fig.1 (a) Bold line: HPLC-ICP MS chromatogram (^{78}Se detection) of SelolTM solution in isopropanol; thin line: Se intensity as a function of the change in the isopropanol/acetonitrile ratio in the eluting solution (no column); (b) the chromatogram in Fig. 1a corrected for the varying sensitivity; (c) HPLC-ICP MS chromatogram of the methanol fraction after partitioning of SelolTM between methanol and hexane.

Fig. 2. *Left panels:* Extracted ion chromatograms (XICs) corresponding to the pseudomolecular ions containing one or more selenium atoms (a) tri-linoleinate (LLL), (b) di-linoleinate-mono-oleate (LLO), (c) mono-linoleinate-di-oleate (LOO) and trioleate (OOO) species. *Right panels:* putative formula supported by MS^2 and MS^3 analysis. The top panels present HPLC-ICP MS chromatogram in order to demonstrate matching of peaks obtained using elemental and molecular MS detection.

Fig. 3. An example of the fragmentation cascade for species m/z 1013.6913 ($\text{C}_{57}\text{H}_{98}\text{O}_8\text{Se}$).

Fig. 4. RP HPLC elution order of the selenized triglycerol $\text{L}_x\text{O}_y\text{S}_z$ (where $x+y+z = 3$) species. The hydrophobicity and the elution time in RP HPLC increase with the decreasing number of double bonds in fatty acid residues; the experimentally established influence of selenization is shown in the inset.

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Table 1. HPLC-ICPMS/ES-MS parameters used.

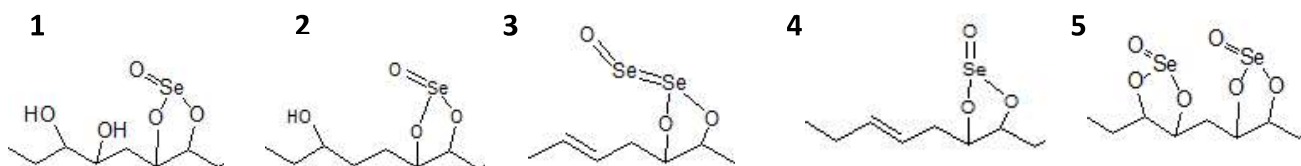
| HPLC | |
|-------------------------|--|
| Column | Zorbax eclipse xdb-C18 (4,6 x 150 mm x 5 µm) |
| Column temperature | 50 °C |
| Injection volume | 500 µl |
| Buffer A | acetonitrile |
| Buffer B | isopropanol |
| Splitter ratio | |
| Flow rate | 0.7 mL/min |
| Gradient | 0-5 min: 0%B, 45-50: 100%B, 55-60:0%B |
| ICP-MS | |
| Mode | Organic mode |
| RF Power | 1550 W |
| RF matching | 2V |
| Nebulizer pump | 0,1 rps |
| Optional gas | 8% |
| Carrier gas | 0.67 L/min |
| He gas | 9.5 mL/min |
| Isotopes monitored | ⁷⁶ Se, ⁷⁷ Se, ⁷⁸ Se |
| ESI-MS | |
| Mode | Positive |
| Capillary Temp [°C] | 350 |
| Source Heater Temp [°C] | 350 |
| Sheath Gas Flow | 75.00 |
| Aux Gas Flow | 20.00 |
| Sweep Gas Flow | 10.00 |
| Source Voltage [kV] | 4.00 |
| Source Current [uA] | 100.00 |
| S-Lens RF Level [%] | 68.00 |

Table 2. Species identification

| formula | mass* | | error, ppm | triglyceride | modification** |
|--|-------------|--------------|------------|--------------|----------------|
| | theoretical | experimental | | | |
| C ₅₇ H ₁₀₀ O ₁₂ Se ₂ | 1159.5437 | 1159,5444 | 0,08 | LLO | 5 |
| C ₅₇ H ₁₀₀ O ₁₁ Se | 1063.6323 | 1063,6337 | 0,79 | LLL | 1 |
| C ₅₇ H ₁₀₀ O ₉ Se | 1031.6425 | 1031.6443 | 1.19 | LLO | 4 |
| C ₅₇ H ₁₀₀ O ₁₀ Se | 1047.6374 | 1047,6389 | 0,96 | LLL | 2 |
| C ₅₇ H ₁₀₀ O ₉ Se ₂ | 1111.5590 | 1111,5594 | 0,34 | LLO | 3 |
| C ₅₇ H ₉₈ O ₉ Se | 1029.6268 | 1029.6290 | 1.62 | LLL | 4 |
| C ₅₇ H ₉₈ O ₈ Se | 1013.6319 | 1013,6325 | -0,01 | OOO | 4 |
| C ₅₇ H ₁₀₂ O ₁₂ Se ₂ | 1049.6536 | 1049,6505 | -2.41 | LLO | 2 |
| C ₅₇ H ₁₀₂ O ₉ Se | 1033.6587 | 1033,6547 | -3.35 | LOO | 4 |
| C ₅₇ H ₁₀₂ O ₉ Se ₂ | 1113.5752 | 1113,5718 | -2.58 | LOO | 3 |
| C ₅₇ H ₁₀₄ O ₉ Se ₂ | 1115.5908 | 1115,5864 | -3.47 | OOO | 3 |

*masses correspond to [M+Na]⁺ except C₅₇H₉₈O₈Se

**modifications :



Figure

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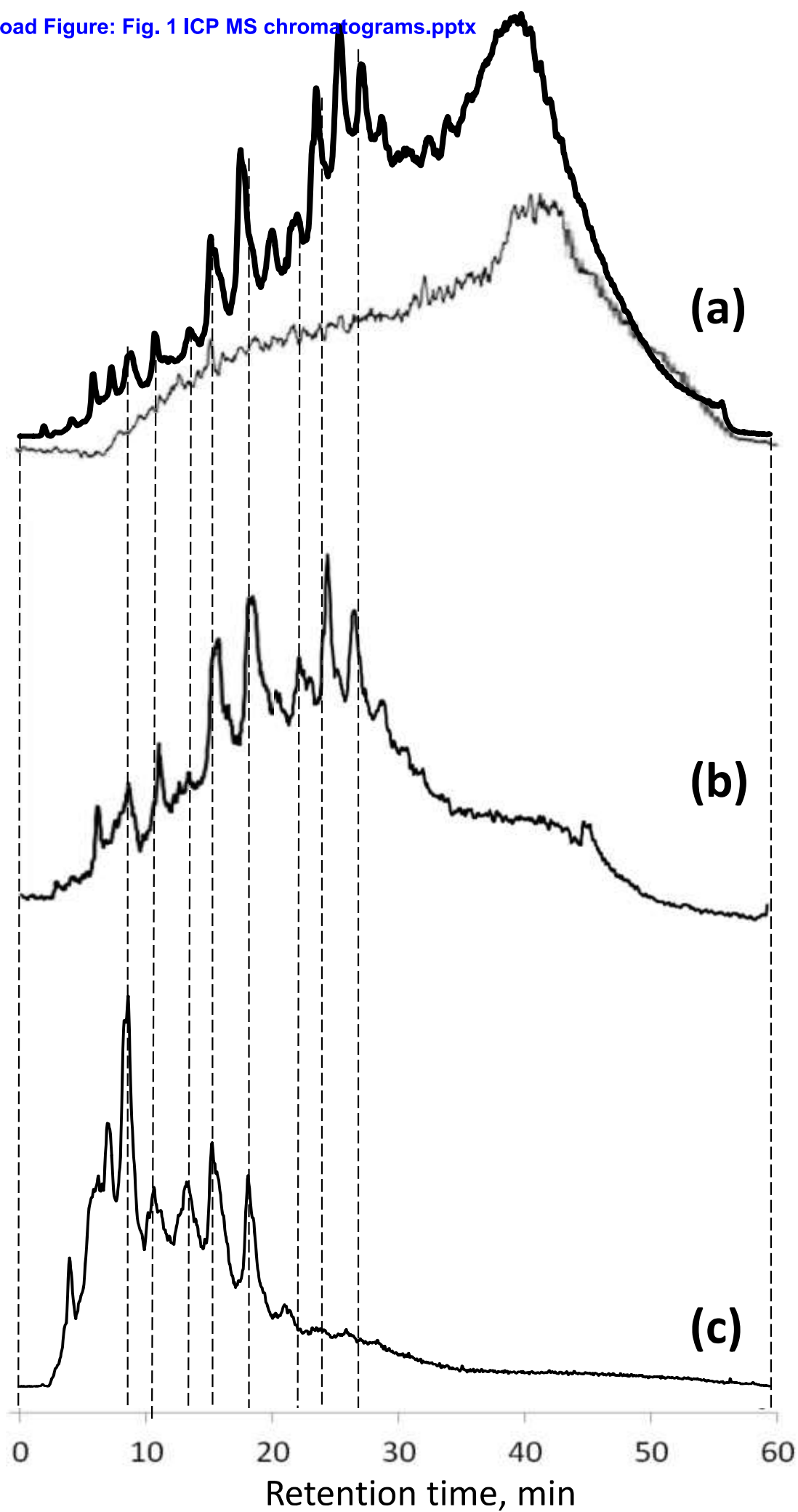
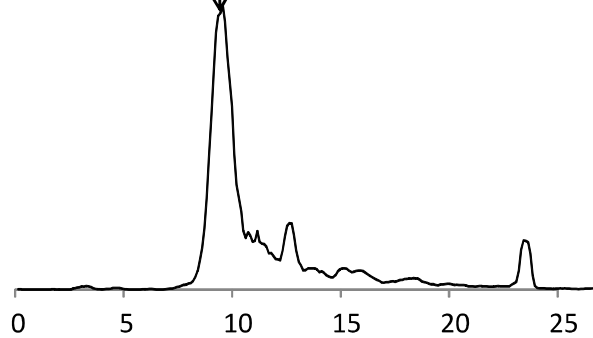
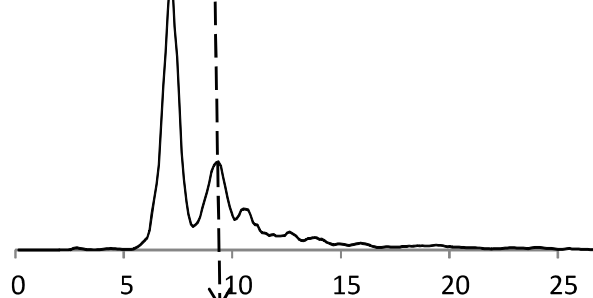
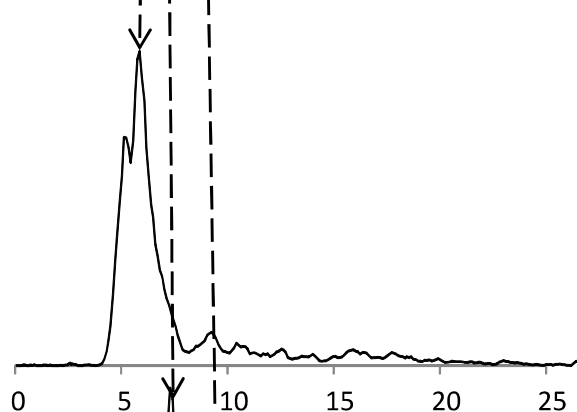
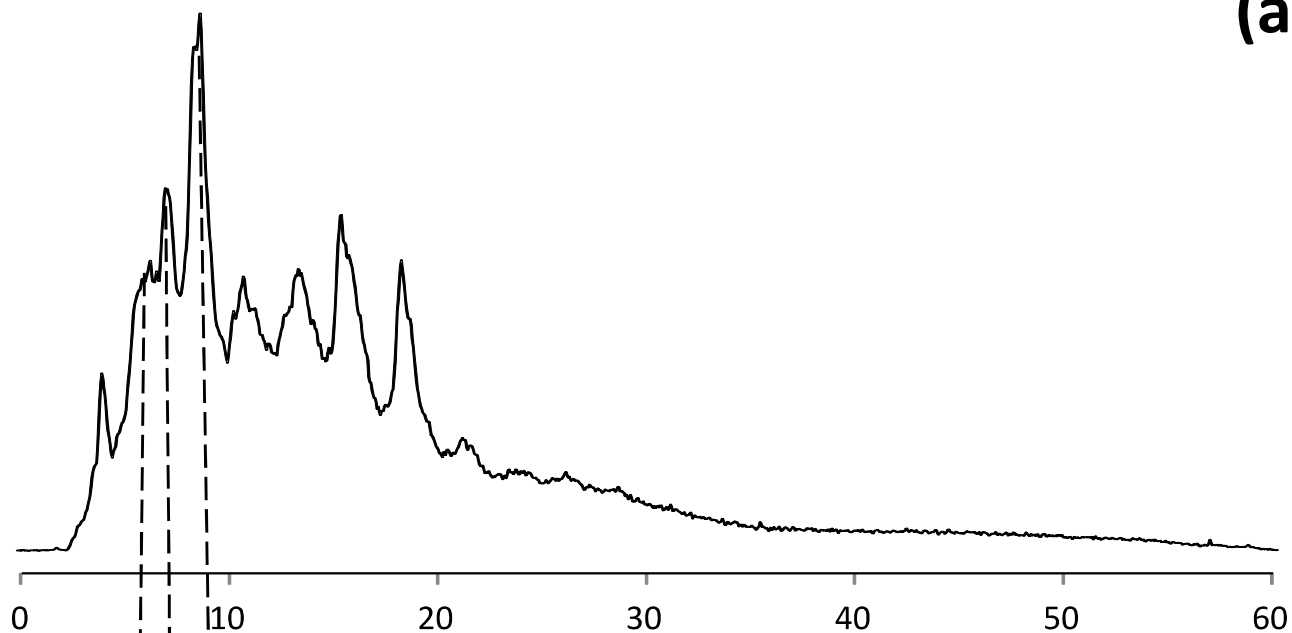
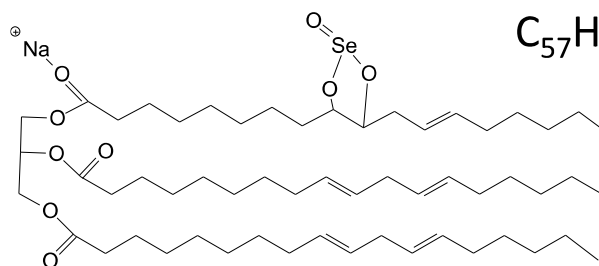
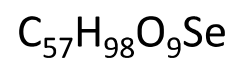
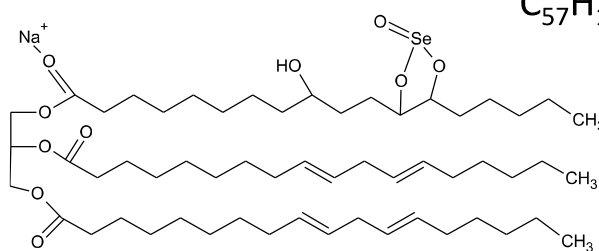
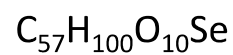
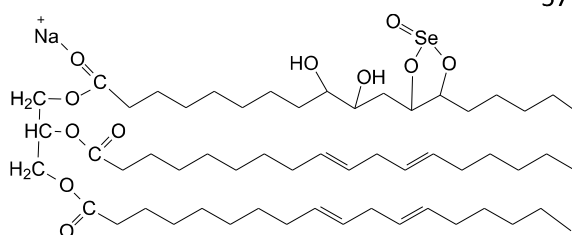
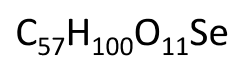


Figure
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(a)

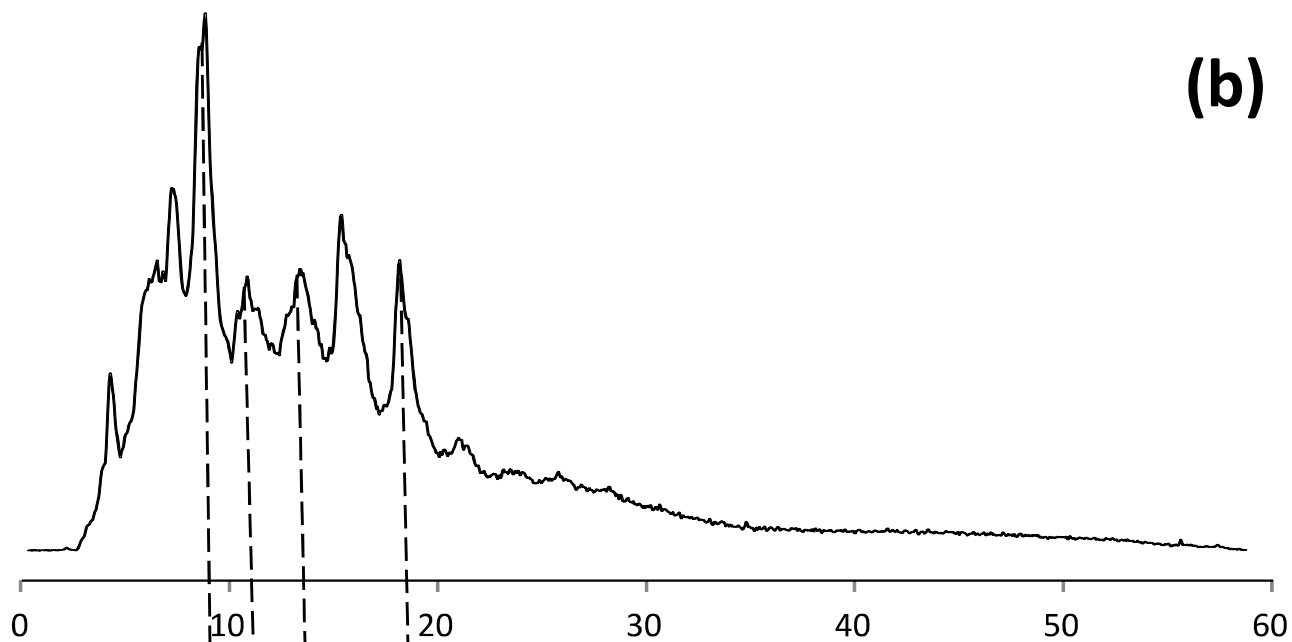


trilinoleate derivatives

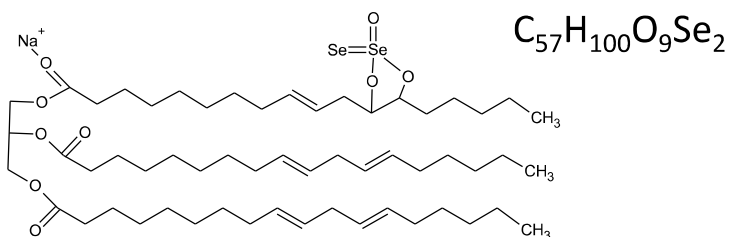
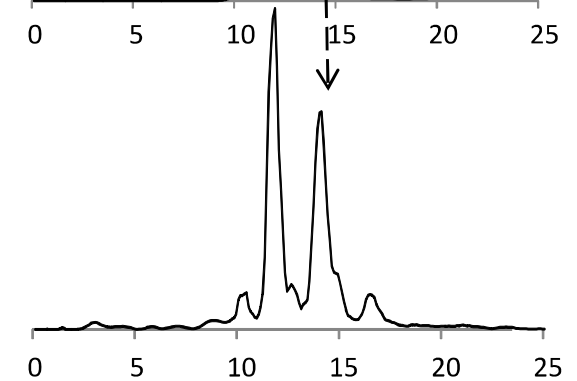
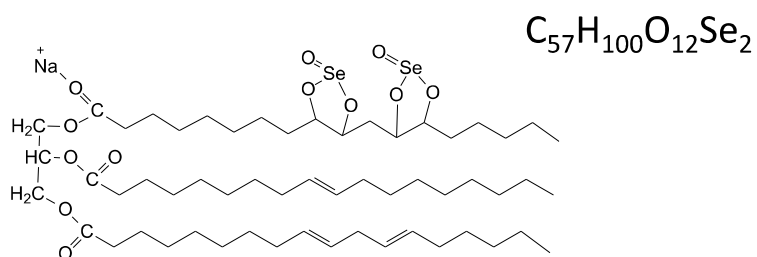
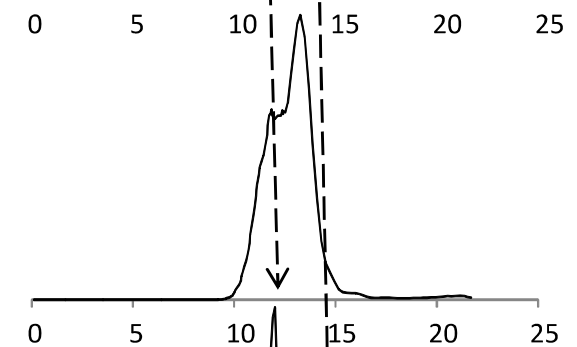
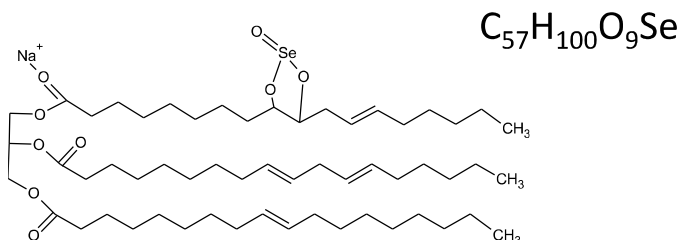
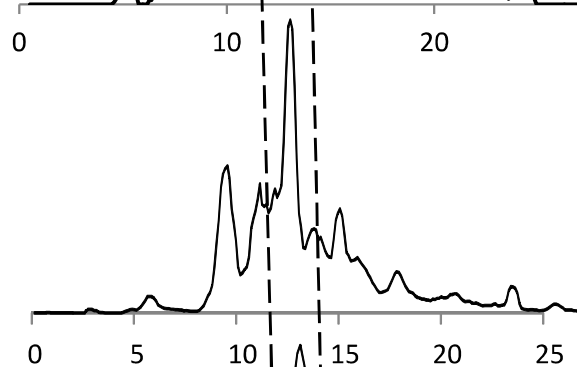
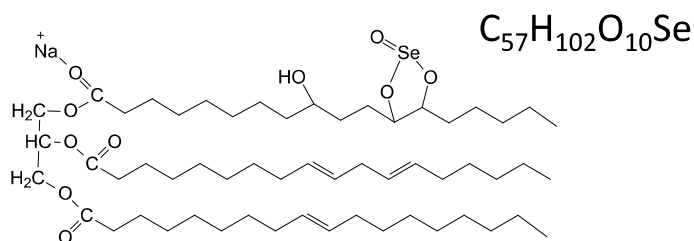
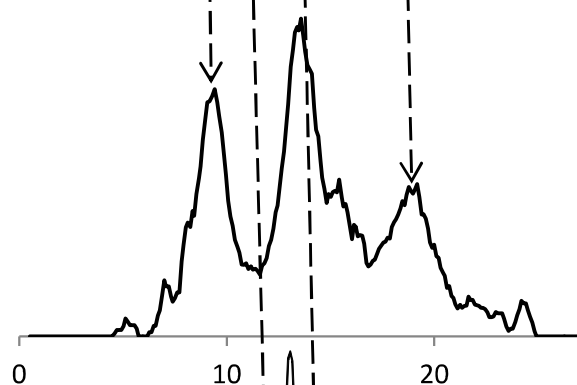


Retention time, min

(b)

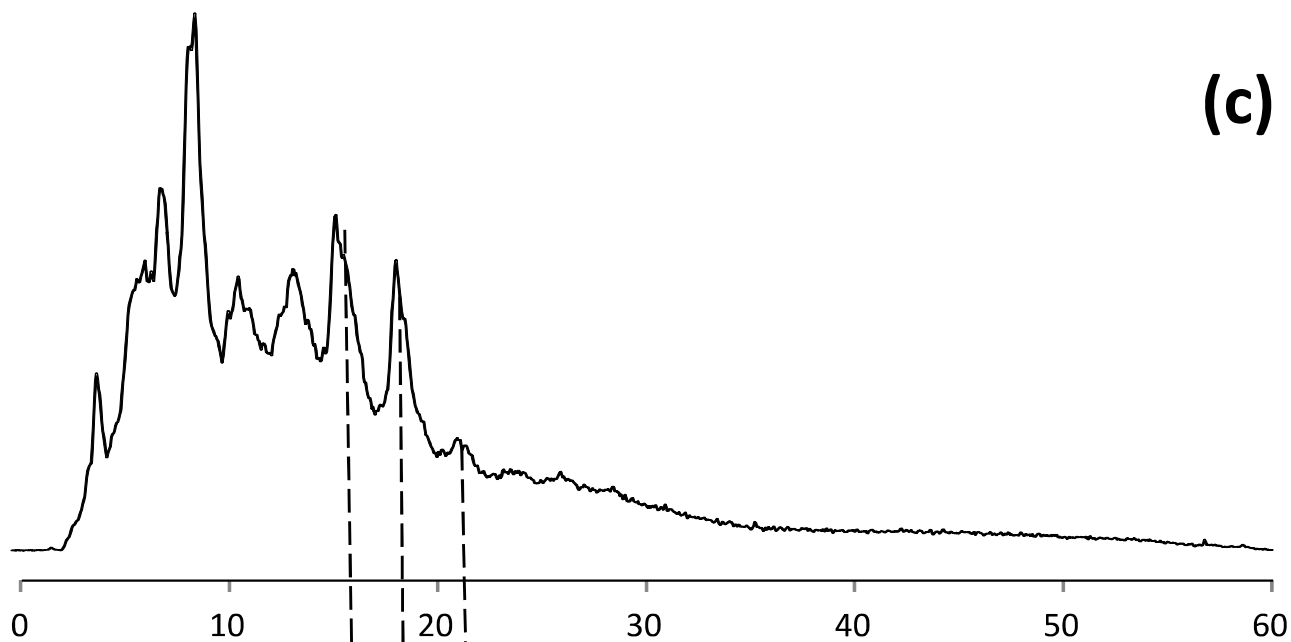


dilinoleate-monooleate derivatives

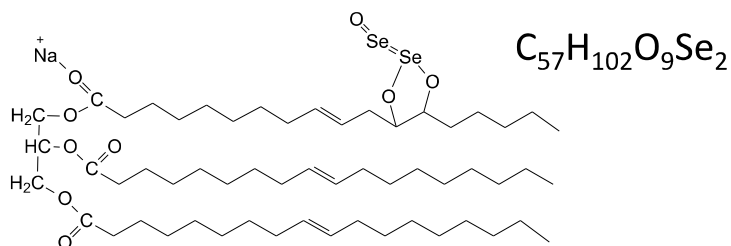
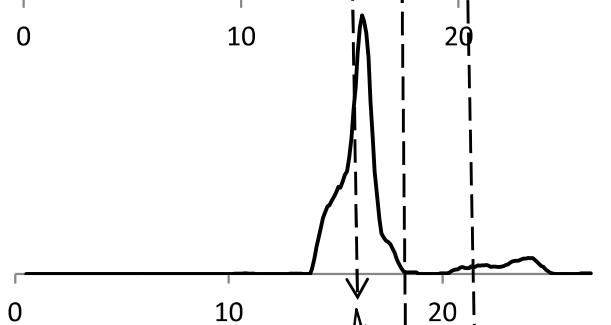
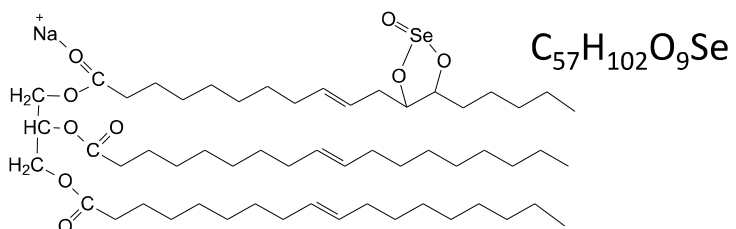


Retention time, min

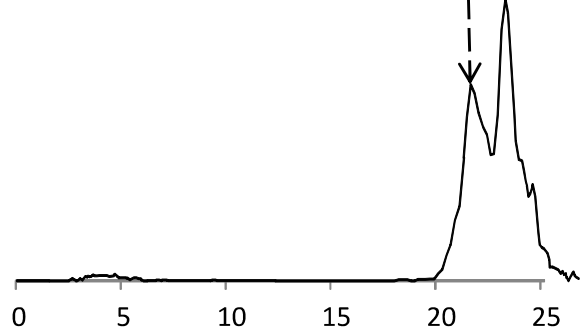
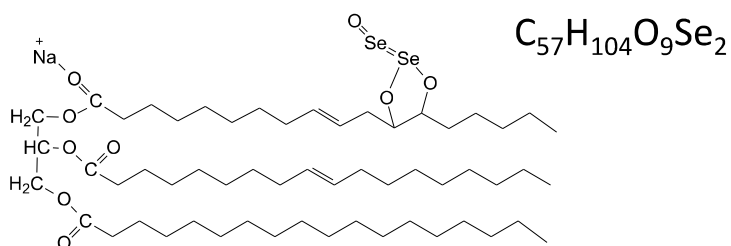
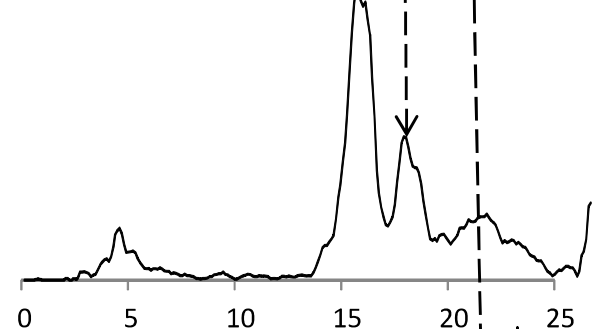
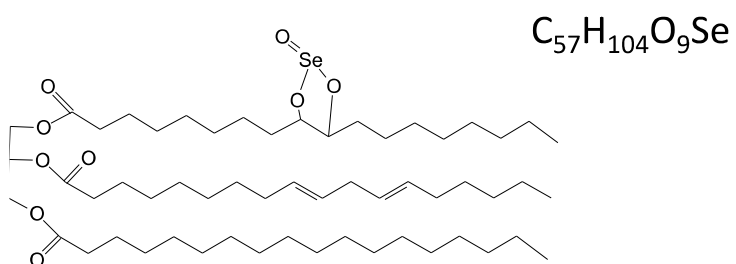
(c)



monolinoleate-dioleate derivatives



trioleate derivative



Retention time, min

Figure

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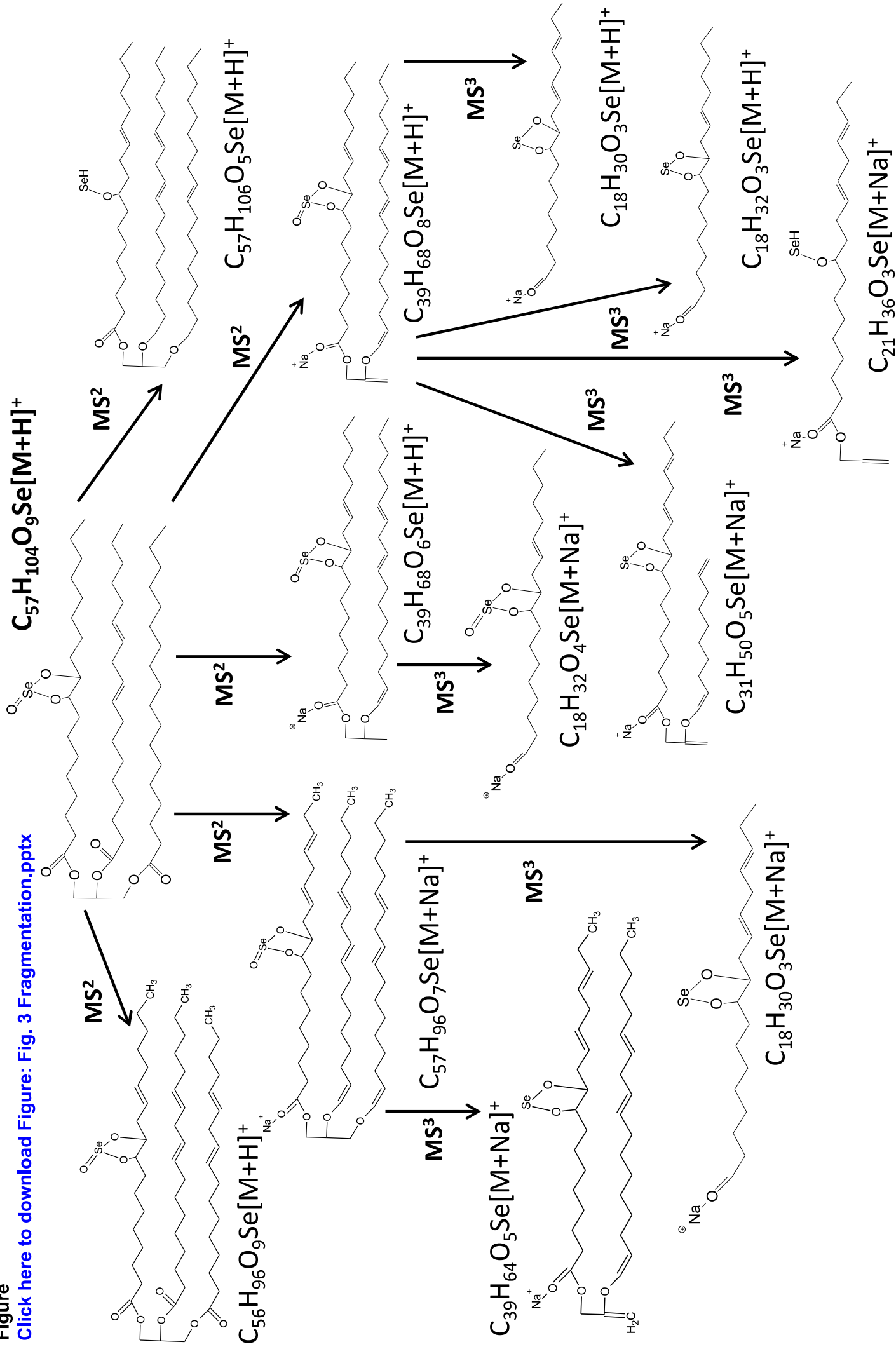


Figure
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