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1 **Identification of crepenynic acid in the seed oil of *Atractylodes lancea* and *A. macrocephala*.**

2

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11

1 **Abstract**

2 Atractylodes rhizome is widely used in traditional Chinese herbal medicine. Although the chemical
3 composition of the root has been studied in detail, the oil content and fatty acid composition of the
4 seeds of *Atractylodes* species have not been reported. Fatty acyl composition of seeds from *Atractylodes*
5 *lancea* and *A. macrocephala* was determined by gas chromatography and mass spectrometry of fatty
6 acid methyl esters and 3-pyridylcarbinol esters. The predominant fatty acid in the seeds of both species
7 was linolenic acid, but the unusual acetylenic fatty acid crepenynic acid (*cis*-9-octadecen-12-ynoic acid)
8 was also observed at levels of 18% in *A. lancea* and 13 to 15% in *A. macrocephala*. Fatty acid content
9 was 24% for the sample of *A. lancea* and 16-17% for seed samples from *A. macrocephala*. *sn*-1,3
10 regioselective lipase digestion of seed lipids revealed that crepenynic acid was absent from the *sn*-2
11 position of the seed triacylglycerol. Crepenynic acid was also found in the seed oil of *Jurinea mollis* at
12 24% and was not present in the *sn*-2 position of the TAG. A contrasting distribution of crepenynic acid
13 was found in the oil of *Crepis rubra*, suggesting differences in crepenynic acid synthesis or TAG assembly
14 between these species.

15

16 **Keywords**

17 *Atractylodes*, *Jurinea*, Crepenynic acid, Acetylenic, Seed oil, *sn*-2 MAG.

18

19 **Abbreviations**

20 FAME(s) Fatty acid methyl ester(s)

21 TAG Triacylglycerol

22 MAG Monoacylglycerol

23

24

Fatty Acid Nomenclature

X:Y$^{\Delta Z}$ Where X is the chain length, Y is the number of double bonds and $^{\Delta Z}$ is the double bond position relative to the carboxyl end of the molecule.

Introduction

Atractylodes rhizome is widely used in traditional Chinese herbal medicine with black atractylodes rhizome, from *Atractylodes lancea* (Thunb.) DC (Cang Zhu, 苍术) being favored for treatment of rheumatic and gastrointestinal ailments, night blindness and influenza, while white atractylodes rhizome, from *A. macrocephala* Koidz. (Bai Zhu, 白术) is more commonly used for treatment of diseases related to the blood and spleen [1,2]. Extensive studies have been conducted to characterize the chemical composition of the rhizomes of both species and a range of potential bioactive secondary metabolites have been identified including sesquiterpenoids, polyacetylenes and monoterpenes [3-7]. Although the roots of *Atractylodes* species have received much attention, seed acyl composition has not been reported. *A. lancea* and *A. macrocephala* are members of the Compositae (Asteraceae), a diverse plant family in which the presence of uncommon fatty acids in seed oil is often a characteristic feature of a genus. [8,9]. Certain uncommon fatty acids are of potential interest as renewable feedstocks for the oleochemical industry, or have value in human health and nutraceutical applications [10]. Epoxidized fatty acids for example are used extensively in adhesives and coatings and plants in the genus *Vernonia* (Compositae) are being evaluated as a potential commercial source of natural epoxy fatty acids [11]. Gamma-linolenic acid (*cis*-6, *cis*-9, *cis*-12-octadecatrienoic acid, GLA, 18:3$^{\Delta 6,9,12}$) is considered beneficial for human health [12] and in the Compositae has been found in the seed oil of *Saussurea* species [13]. As the presence of novel fatty acids with commercial potential in the seed oil of *Atractylodes* could offer additional value to the crop, we determined the seed fatty acid content and composition of samples of *A. lancea* and *A. macrocephala*.

Experimental Procedures

Seed

Seed of *A. lancea* and *A. macrocephala* was purchased from Xuxin Pharmaceutical Sales Co., Ltd., Bozhou, Anhui, China (http://bzxxzy.com). An additional sample of *A. macrocephala* and seed of *Crepis rubra,* and *Jurinea mollis* were purchased from B & T World Seeds, Paguignan, 34210 Aigues-Vives, France (B+T, www.b-and-t-world-seeds.com). Names and synonyms were validated by referencing The Plant List (www.theplantlist.org).

Extraction of seed oil and separation by TLC.

Dry seeds (30-50 mg) were placed in glass tubes containing 2 ml of hexane and crushed using a glass rod. Tubes were centrifuged at 2000 x g for 2 minutes to precipitate solid material. After transfer to a fresh tube, the hexane was evaporated under a stream of nitrogen gas and lipids were taken up in 200 μl of hexane. Lipids were separated by thin layer chromatography (TLC) using Silica Gel G60 aluminium backed plates (Whatman Ltd, Kent, England) with hexane/diethyl ether/acetic acid (140:60:2 by volume) as solvent. Lipids were visualized using phosphomolybdic acid-oxidizing stain as described previously [14].

Preparation of FAMEs and 3-pyridylcarbinol esters and analysis by GC and GC-MS

Prior to analysis, seed samples were carefully inspected to remove foreign material such as dirt and other plant parts. For analysis by direct acid catalyzed transmethylation, seeds were crushed and four individual samples (25-30 mg) from each species were weighed to determine dry weight. Total lipids were transmethylated *in-situ* by refluxing for 16 hours at 80°C in glass tubes containing 2 mL 1M HCl in methanol with 100 ug of 17:0 FAME and 500 μL of hexane. After cooling, 2 mL of 0.9% NaCl was added

1 and 200 μL of the hexane phase was transferred to a glass GC vial. For base catalyzed transesterification,
2 2 ml of 0.5 N methanolic base (Supelco, PA, USA) was used with a 2 hour incubation at 80°C. FAMES
3 were separated by GC using an Agilent 6890N GC equipped with a DB-23 capillary column (0.25 mm x 30
4 m, 0.25 μm thickness; J&W; Folsom, CA, USA) and a flame ionization detector with Helium as carrier gas.
5 Analysis was conducted in split mode (50:1) with a 2 μl sample injection and inlet temperature of 240^{0}C.
6 Initial column temperature was 160°C, held for 1 min then increased to 240^{0}C at 4^{0}C/min. Column
7 temperature was held at 240°C for 10 minutes to ensure that all FAMES passed through the column. For
8 GC-MS analysis of FAMES, an Agilent 7890A GC equipped with an identical 30 m DB-23 capillary column
9 and an Agilent 5975C mass selective detector was used. Analysis was conducted in split mode (40:1)
10 with an inlet temperature of 240^{0}C. Initial column temperature was 120°C, held for 1 min then increased
11 to 240^{0}C at 4^{0}C/min and held at 240°C for 10 minutes. For analysis of 3-pyridylcarbinol esters, seed lipids
12 were extracted as described above and saponified using 0.1 M potassium hydroxide in 90% aqueous
13 ethanol. Free fatty acids were converted to 3-pyridycarbinol esters by first converting to an imidazolide
14 intermediate then reacting with 3-(hydroxymethyl)pyridine in the presence of trimethylamine as
15 previously [15]. GC-MS was conducted using an Agilent 6890N gas chromatograph equipped with a
16 5973N mass selective detector and an MXT-1 capillary column (0.28mm x 15 m, 0.25μm thickness;
17 www.restek.com). The initial oven temperature of 160°C held for 1 minute, then increased to 300°C at a
18 rate of 2^{0}C/min.

19 ***sn*-1,3 regioselective lipase digestion of seed lipids**

20 Hexane extracted seed lipid was transferred to a 16 ml glass screw top tube and the hexane was
21 removed under a stream of nitrogen gas. Lipids were re-dissolved in 250 μl of diethyl-ether and 1 ml of
22 buffer (0.1 M Tris HCl pH 7.7 with 5 mM $CaCl_2$) was added followed by 15 μl of *Rhizomucor miehei* lipase
23 (Sigma L4277). Tubes were capped and incubated with shaking in a Thermo-Shaker (MBI lab equipment,
24 Dorval, PQ, Canada) at 37°C for 90 minutes to achieve digestion of approximately 75% of the TAG. The

reaction was stopped by addition of 100 μl 0.15 M acetic acid and 3.5 ml of chloroform/methanol (2:1 by volume). After centrifugation at 750 x g for 1 minute, lipids were recovered in the chloroform phase, transferred to a fresh tube and the chloroform was evaporated under a stream of nitrogen gas. Lipids were re-dissolved in hexane for separation by TLC was conducted using Silica Gel G60 plates with hexane/diethyl ether/acetic acid (80:120:2 by volume) as solvent. Lipids were applied to the TLC plates in bands and the location of the digestion products after TLC was achieved by cutting off the edges of the bands and staining these strips using iodine vapour. Lipids were then recovered from the remaining untreated parts of the TLC plate by scraping the appropriate regions of the silica gel into 16 ml glass screw top tubes. FAMES were prepared by direct transmethylation of the silica gel using 3 ml of 1 M HCl in methanol with 500 μl hexane and incubation at 80°C for 90 minutes. After cooling and addition of 3 ml 0.9% NaCl solution, FAMEs were recovered in the hexane phase for analysis by GC as described above.

Results and discussion.

Separation of the hexane extracted seed lipids of *A. macrocephala* by TLC indicated that the primary component was triacylglycerol (Fig. 1a). To determine seed acyl composition, seeds were directly transmethylated and FAMEs were separated by GC. The predominant fatty acids in both *Atractylodes* species were common 16-carbon and 18-carbon (C-16 and C-18) fatty acids with linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid, 18:2$^{\Delta 9,12}$) being most abundant at around 60% (Table 1). Chromatograms from both species also contained an additional peak with a retention time close to that of the C-20 fatty acids (Fig. 2b). The mass spectrum of the FAME of this unknown component gave a low abundance molecular ion at 292.3 *m/z* and fragmentation pattern characteristic of methyl crepenynate (*cis*-9-octadecen-12-ynoic acid methyl ester) [16,17]. To confirm the location of the double and triple bonds, fatty acids prepared from the oil of *A. macrocephala* were converted to 3-pyridylcarbinol esters. From the mass

1 spectrum, the $[M-1]^+$ ion at 364.8 m/z for the unknown compound confirmed the mass of the ester
2 while fragment ions pairs at 298.1 *m/z* and 274.1 *m/z* and at 234.1 *m/z* and 260.1 *m/z* enabled location
3 of the double and triple bonds at C-9 and C-12 respectively (Fig. 2b). For further confirmation, we
4 obtained seed of *Crepis rubra*, which was reported to contain 55% crepenynic acid in its seed oil [18].
5 Mass spectra of the methyl and 3-pyrindylcarbinol esters of crepenynic acid from this species matched
6 those from the *Atractylodes* samples (data not shown). Crepenynic acid accounted for almost 18% of
7 total seed fatty acids in the single sample of *A. lancea* examined, with levels being slightly lower in both
8 samples of *A. macrocephala*. Noticeable variation in seed fatty acid composition was seen between the
9 *A. macrocephala* samples. More extensive analysis is clearly needed before it can be determined
10 whether differences in crepenynic acid content are a distinguishing feature for the two species.

11 Presence of crepenynic acid in the genus *Crepis* is often accompanied by the presence of epoxy fatty
12 acids such as vernolic acid (12-13-epoxy-*cis*-9-octadecenoic acid, 12-epoxy-$18{:}1^{\Delta 9}$) in the seed oil [18]. As
13 epoxy fatty acids may be labile under the conditions used in acid catalyzed transmethylation, seed oil
14 from both *Atractylodes* species was transmethylated under basic conditions using sodium methoxide.
15 No evidence of epoxy fatty acids was found.

16 The unusual acetylenic fatty acid crepenynic acid has been observed in the seed oil of a range of species
17 [8,9,19] and its pathway of biosynthesis has been extensively characterized in members of the genus
18 *Crepis*. Early studies using developing seed of *Crepis rubra* established that crepenynic acid was
19 synthesized by the introduction of a triple bond (acetylenic bond) into an existing fatty acid, with the
20 nearest precursor being oleic acid (*cis*-9-octadecenoic acid, $18{:}1^{\Delta 9}$) [20]. The fatty acid acetylenases of
21 several species of *Crepis* were subsequently cloned and found to be divergent members of the plant
22 "fatty acid desaturase 2" (FAD2) family catalyzing the extraction of the two remaining hydrogen atoms
23 from an existing double bond between C-12 and C-13 of linoleic acid [21,22]. The lipid substrate for

acetylenation was tentatively identified as the membrane phospholipid phosphatidycholine (PC) [23]. Evidence for bifunctional acetylenase/desaturase activity was also reported with enzyme from *C. alpina* being able to desaturate oleate [24]. Studies on the stereospecific distribution of crepenynate in seed oil from *C. alpina* by partial hydrolysis using pancreatic lipase indicated that a large proportion of the crepenynic acid was esterified in the *sn-2* position of TAG [20]. This was later confirmed by a combination of HPLC and NMR [25]. To examine crepenynate distribution in *Atractylodes* we conducted *sn*-1,3 regioselective lipase digestion of seed lipids from both species using a commercial lipase from *Rhizomucor miehei*. As a control we also extracted and treated a sample of oil from *Crepis rubra*. As shown in Figure 3, the acyl composition of *sn*-2 MAG produced from the *Atractylodes* samples was very different to that from *C. rubra*. In both *Atractylodes* species, crepenynic acid was largely absent from the *sn*-2 position (Table 1), whereas this fatty acid accounted for 55% of the fatty acids in *C. rubra sn*-2 MAG. To extend our studies we screened our collection of species in the Compositae and identified crepenynic acid in the seed oil of *Jurinea mollis* (Table 1). This fatty acid accounted for 24.1% of total seed fatty acids, but similar to the *Atractylodes* samples, crepenynic acid was largely absent from the *sn-2* position of TAG (Table 1). The contrasting distribution of this unusual fatty acid in TAG suggests that differences in the pathways of crepenynic acid biosynthesis and incorporation into TAG exist in these species. Characterization of TAG biosynthesis in these plants would be of interest for those attempting to produce acetylenic fatty acid production in genetically engineered oilseeds.

In our analysis of FAMEs prepared from the seed oil of *C. rubra*, crepenynic acid accounted for 29.5% of total seed fatty acids, considerably lower than the 55% previously reported [18], with $18{:}2^{\Delta 9,12}$ being the most abundant fatty acid. In addition, a number of minor components were observed totaling close to 7% of total seed fatty acids (Table 1). Due to lack of sufficient seed material, and because this species was not the focus of our work we did not attempt to identify all of these. One component however was

observed with a retention time close to that of linoleic acid and received further investigation (Fig.3(b), peak a). The mass spectrum of the FAME (data not shown) was identical to that of methyl linoleate ($18{:}2^{\Delta 9,12}$) with a characteristic molecular ion at 294.2 *m/z* and an $[M\text{-}31]^+$ ion at 263.1 *m/z* accompanied by an $[M\text{-}32]^+$ ion of lower intensity. This fatty acid was most obvious in the MAG fraction after lipase digestion (Fig. 3(b)). Based on previous observations made during the characterization of the bifunctional acetylenase/desaturase from *C. alba*, [24] a speculative identity could be $18{:}2^{\Delta 9cis,\ \Delta 12\ trans}$ a product of the desaturase activity of the *C. rubra* acetylenase on endogenous oleate.

Although polyacetylenes and polyacetylene glycosides are considered to be important bioactive components of atractyloides rhizome [5,7] and of many other traditional medicinal plants and fungi [26,27], their primary role in plants may be defense. Acetylenic acids and their derivatives have been shown to be potent antifungal and insecticidal agents and can also function as allelochemicals, inhibiting the growth of competing plants [27,28]. Acetylenic secondary metabolites are largely derived from acetylenic fatty acids including crepenynic acid and acetylenase genes appear to be widespread in the plant kingdom, although most plants to not produce acetylenic acid in their seed oil [17]. Characterization of acetylenase genes and their expression in *Atractylodes* would be a method to assess whether the pathways of crepenynic acid biosynthesis in the seed and polyacetylene synthesis in the root are inter-related.

Acetylenic fatty acids are known to act as irreversible inhibitors of lipoxygenase enzymes, through the activity of hydroperoxides formed during their oxidation [29]. Crepenynic acid in the diet has been shown to cause acute muscular breakdown and mortality in sheep and chicks [30]. The toxic nature of this fatty acid to livestock was first observed through a study on mortality in Western Australian sheep grazing on pasture rich in mature seeded plants of *Ixiolaena brevicompta* (a synonym of *Leiocarpa brevicompta* (F. Muell.) Paul G. Wilson). Analysis of the seed oil of this species revealed a crepenynic

acid content of 25% and an oil content of up to 12% [31]. The presence of this fatty acid in *Atractylodes* suggests that the seeds and oil of these plants may not be suitable for consumption by humans or livestock.

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Tables

Table 1. Seed fatty acid content and composition.

Figure legends

Figure 1. **(a)** Separation of seed lipids by TLC. Track 1, lipids from *A. macrocephala*; track 2, reference TAG (seed lipids from *Arabidopsis thaliana*). (b) Gas chromatogram showing separation of FAMEs prepared from mature seed of *A. macrocephala*. Peaks were identified by comparison to standards and confirmed by GC-MS, a = methyl-palmitate (16:0), s = internal standard, methyl heptadecanoate (17:0), b = methyl stearate (18:0), c = methyl oleate ($18{:}1^{\Delta 9}$) , d = methyl linoleate ($18{:}2^{\Delta 9,15}$), e = methyl crepenynate (12-acetylenic-$18{:}1^{\Delta 9}$).

Figure 2. Mass spectra of (a) methyl crepenynate prepared from seed oil of *A. macrocephala,* (b) 3-pyridylcarbinol ester of crepenynic acid prepared from seed oil of *A. macrocephala*. Diagnostic ions (in bold) are indicated.

Figure 3. Gas chromatograms of FAMEs prepared from *sn*-2 MAG derived from lipase digestion of seed oil from (a) *Atractylodes lancea*, (b) *Crepis rubra* (peak b not unidentified).

		Fatty acid (%)										
Sample	**Seed fatty acid content (%)**	**14:0**	**16:0**	**18:0**	**$18:1^{\Delta9}$**	**$18:1^{\Delta11}$**	**$18:2^{\Delta9,12}$**	**$18:3^{\Delta9,12,15}$**	**20:0**	**$20:1^{\Delta11}$**	**Crepenynic**	**Other**
Atractylodes lancea	24.1		7.0	4.5	11.0	0.4	58.2	0.4	0.5	0.2	17.8	
Atractylodes macrocephala	16.0		9.5	3.3	9.2	0.5	60.5	0.8	0.6	0.4	15.2	
Atractylodes macrocephala (B+T)	16.8		8.7	4.1	11.5	0.4	61.2	0.6	0.7	0.2	12.6	
Jurinea mollis (B+T)	17.0	1.2	9.0	1.9	23.9	0.8	37.2	0.2	0.7	0.2	24.1	0.8
Crepis rubra (B+T)	ND		10.3	7.7	8.3	0.8	32.6		3.1	0.4	29.5	7.3
A. lancea sn-2 MAG			0.5	0.1	9.0		89.5	0.1			0.7	
J. mollis sn-2 MAG			1.2	0.5	41.3		54.3	1.1		0.4	1.2	
C. rubra sn-2 MAG			1.6	1.6	8.7		31.9 *				55.2	1.0

ND = not determined.

*Total 18:2 = 26.7% $18:2^{\Delta9,12}$ plus 5.2% 18:2-isomer.

Figure 1

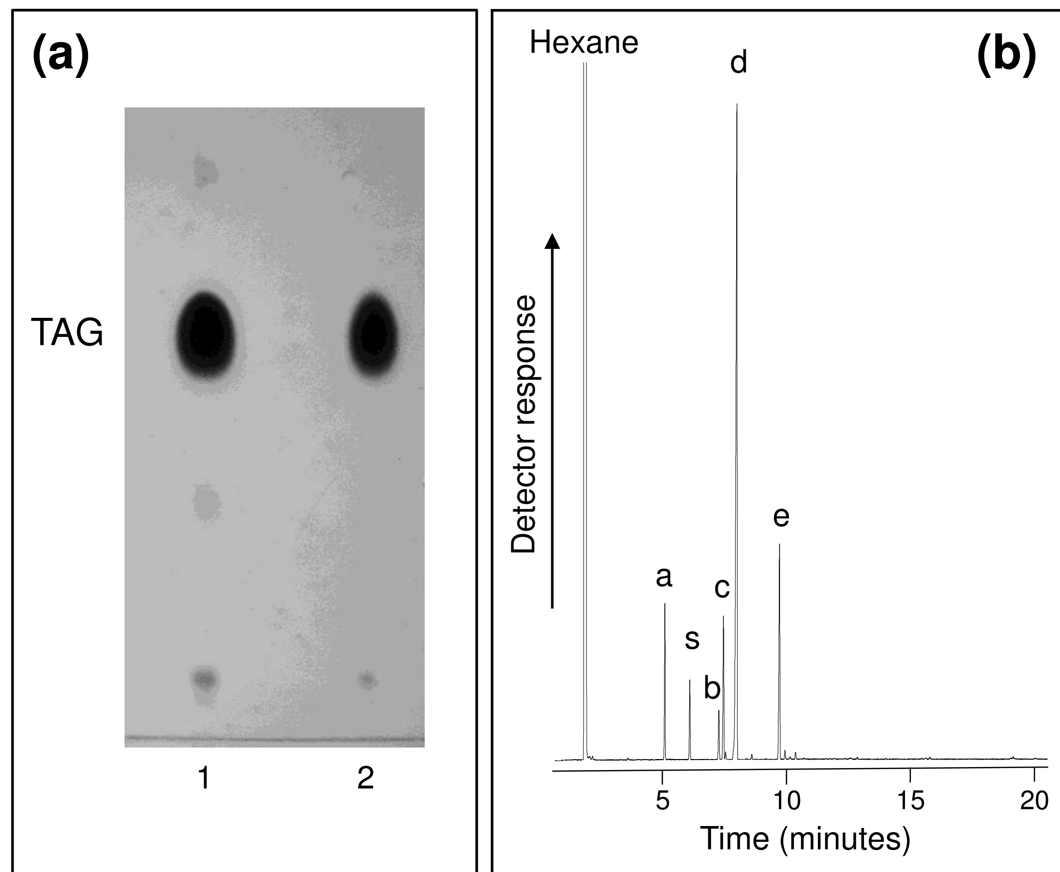
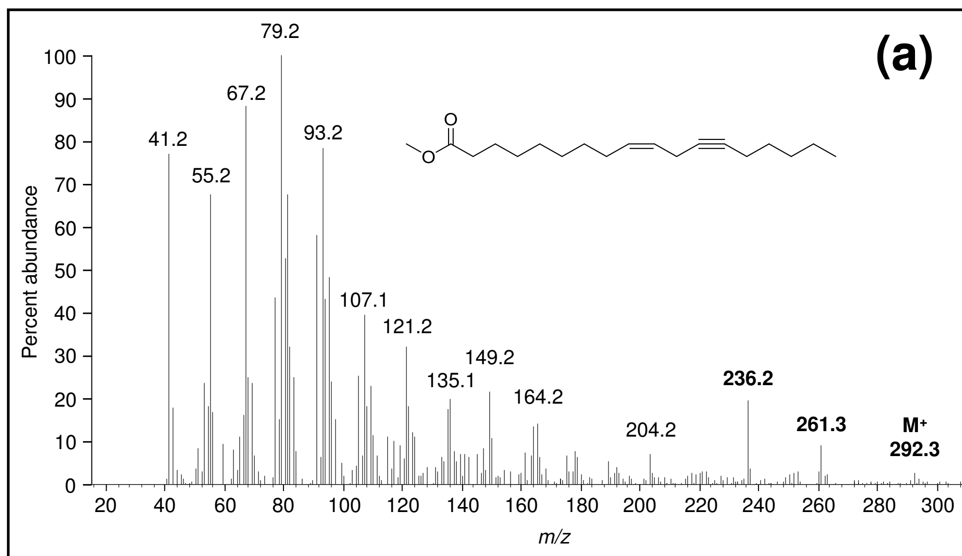


Figure 2



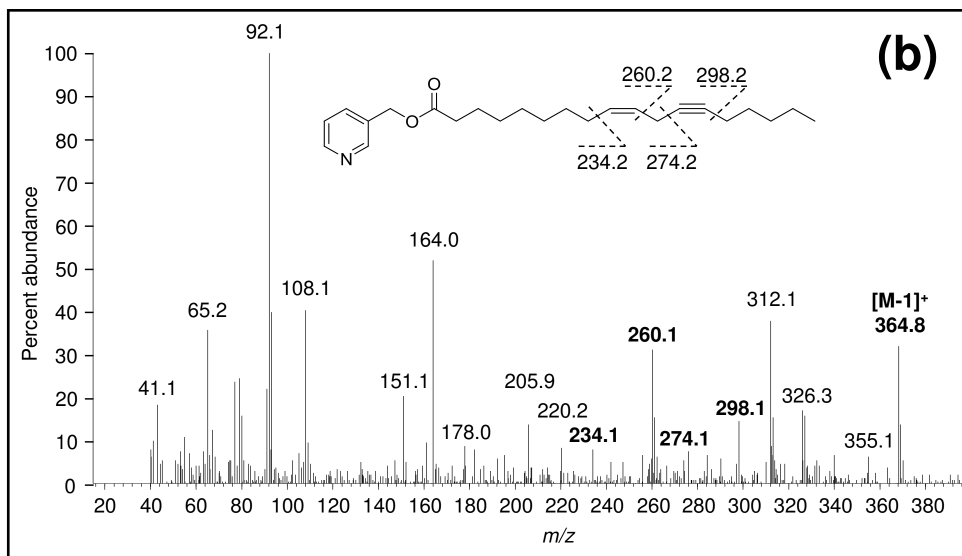


Figure 3