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Article

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A Gas Chromatography-Mass Spectrometry (GC-MS) Method for the Detection and Quantitation of Monofluoroacetate in Plants Toxic to Livestock

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ABSTRACT

Monofluoroacetate (MFA) is a potent toxin that occurs in over 50 plant species in Africa, Australia, and South America and is responsible for significant livestock deaths in these regions. A gas chromatography–mass spectrometry (GC-MS) method for the analysis of MFA in plants based on the derivatization of MFA with *n*-propanol in the presence of sulfuric acid to form propyl fluoroacetate was developed. This method compared favorably to a currently employed high performance liquid chromatography-mass spectrometry (HPLC-MS) method for the analysis of MFA in plants. The GC-MS method was applied to the analysis of MFA in herbarium specimens of *Fridericia elegans, Niedenzuella stannea, N. multiglandulosa, N. acutifolia*, and *Aenigmatanthera lasiandra*. This is the first report of MFA being detected in *F. elegans, N. multiglandulosa, N. acutifolia*, and *A. lasiandra,* some of which have been reported to cause sudden death or that are toxic to livestock.

Keywords: monofluoroacetate (MFA); *Friderica elegans*; *Niedenzuella multiglandulosa*; *Niedenzuella acutifolia*; *Aenigmatanthera lasiandra*; gas chromatography-mass spectrometry (GC-MS)

INTRODUCTION

Monofluoroacetate (MFA) is a naturally occurring toxin found in over 50 plant species distributed through the Fabaceae, Rubiaceae, Bignoniaceae, Malpighiaceae and Dichapetalaceae families in Africa, Australia, and South America. Several *Gastrolobium* and *Dichapetalum* spp. within the Fabaceae and Dichapetalaceae families, respectively, have been documented to contain MFA.[1] The MFA containing *Gastrolobium* species in Australia and the *Dichapetalum* species in Africa have been responsible for significant livestock deaths in these regions with *D. cymosum* reported as the third most important poisonous plant in South Africa.[2,3]

Plants that contain MFA in South America belong to the Rubiaceae, Bignoniaceae, and Malpighiaceae families.[1] These plants poison livestock causing a disease called "sudden death syndrome" which is responsible for half of all cattle deaths in Brazil.[4] Livestock poisoned by MFA containing plants exhibit loss of balance, ataxia, tachycardia and labored breathing, ultimately leading to death shortly after initial clinical signs.[5] *Palicourea marcgravii*, a member of the Rubiaceae family, is the most significant poisonous plant in Brazil because of its palatability, acute toxicity to livestock, and its wide geographical distribution.[4] In addition to *P. marcgravii*, several other *Palicourea* spp., *P. aeneofusca*, *P. barraensis*, *P grandiflora*, *P.* aff. *juruana*, and *P. longiflora* contain MFA,[1,5-7] likewise, in the Malpighiaceae family several *Amorimia* spp., *A. amazonica, A. exotropica*, *A. publiflora*, *A. rigidia*, and *A. septentrionalis*, contain MFA and cause "sudden death".[1,4,8] In both these genera other species have been reported to contain MFA; however, they have yet to be associated with this syndrome.[6,8] Recently, another species within the Malpighiaceae, *N. stannea*, was reported to cause "sudden death" and contain MFA.[9] In the Bignoniaceae, two species (*Tanaecium bilabiatum* and *F.*

elegans) are reported to cause the syndrome,[1,4] where *T. bilabiatum* has been reported to contain MFA.[10] Another Bignoniaceae species, *F. japurensis* was previously thought to cause "sudden death"; however a recent paper demonstrated that the original specimens were misidentified and are correctly classified as the MFA-containing plant *T. bilabiatum*.[11]

Several analytical methods have been developed for the analysis of monofluoroacetate in plants including: colorimetry,[12] ^{19}F NMR spectroscopy,[10,13] ion chromatography with conductivity detection,[14] HPLC with UV detection,[15] gas chromatography–mass spectrometry (GC-MS) with derivatization of MFA with pentafluorobenzyl bromide,[16] and, high performance liquid chromatography-mass spectrometry (HPLC-MS).[8]

Monofluoroacetate in the form as the sodium salt, also known as "Compound 1080" is extremely toxic and has been used in many countries as a rodenticide and for the control of predators. The wide historical and more regulated current use of sodium monofluoroacetate for the control of pests has resulted in the development of various analytical methods for analysis of sodium monofluoroacetate in baits, serum, urine, animal tissues and foods. Most recently, HPLC-MS methods have been developed in response to threats by environmental activists to contaminate milk and infant formulas with sodium monofluoroacetate.[17-19] Several gas chromatography (GC) methods have been developed for the analysis of sodium monofluoroacetate in these matrices. One report describes the GC-MS analysis of residues of sodium monofluoroacetate on sheep wool/skin as the free acid, however, the authors describe the persistence of "ghost peaks" in blank samples after analysis of MFA containing samples due to adsorption of MFA in the GC injection port and at the head of the GC column.[20] Because MFA is highly polar and relatively non-volatile, successful GC analysis has, in general, required derivatization prior to analysis.

Derivatization prior to final analysis can also add selectivity in that many of the compounds extracted from a matrix will not react with the derivatizing agent and thus, will not contribute to interferences in the final analysis. Monofluoroacetate has been analyzed in water, beverages, baits, animal stomach contents, blood, plasma, serum, urine, animal and human tissues by GC after derivatization with a variety of dervatizing agents including: pentafluorobenzyl bromide;[21-25] 2,4-dichloroaniline;[26] 1-pyrenyldiazomethane;[27] benzyldimethylphenylammonium hydroxide;[28] diazomethane;[29] ethanol;[30,31] and ethanol and *n*-propanol.[32]

Due to the significance of livestock losses from MFA containing plants around the world and because of the availability of GC-MS in many laboratories an uncomplicated, inexpensive, GC-MS method for the analysis of MFA in plant samples was developed. Only one report of the analysis of plant material for MFA with GC-MS has been reported.[16] The method described herein is based on the extraction of MFA from the plant matrix, reaction of *n*-propanol with the extracted MFA, and final GC-MS analysis of the propyl fluoroacetate (PFA). Using this method herbarium voucher specimens of *F. elegans*, a plant reported to cause sudden death, were analyzed for MFA. In addition, several taxa morphologically and/or phylogenetically related to *N. stannea*,[33,34] a species recently reported to contain MFA,[9] were analyzed for MFA. We suspected that they may contain MFA as some of these taxa cause clinical signs in livestock similar to those observed in sub-lethal doses of MFA-containing taxa that cause sudden death.[4]

MATERIALS AND METHODS

Chemical and Reagents. Sodium monofluoroacetate (SMFA) and sodium monochloroacetate (SMCA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-propanol and anhydrous

sodium sulfate were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ), sulfuric acid was obtained from (Pharmacy Products (Brookfield, CT) and chloroform was purchased from Fisher Scientific (Pittsburg, PA). Pure water (18.2 MΩ/cm) was obtained from a WaterPro PS Station (Labconco, Kansas City, MO).

Standard solutions. Calibration standards were prepared in water from SMFA and SMCA. Stock solutions of 100 μg/mL of SMFA and SMCA were prepared. Each stock solution was diluted resulting in working solutions of 2 μg/mL. All of the stock and working solutions were stored at 4 °C. Aliquots of the working solutions were used to prepare the calibration curve for SMFA.

Plant material. Collections of *A. septentrionalis* were made in the state of Paraíba, Brazil at two locations (S7° 09.47 W37° 19.06; elevation 305 m; S7° 12.24 W37° 15.11; elevation 749 m). Voucher specimens were deposited at the USDA-ARS Poisonous Plant Research Laboratory Herbarium.[7] Individual *A. septentrionalis* plants were separated into leaves, stems, and floral parts, air dried and ground to pass through a 2 mm screen. After grinding the individual plants and plant parts, a portion of the ground samples were combined and mixed thoroughly by repeatedly inverting the container for 24 h to create a composite *A. septentrionalis* sample. *P. marcgravii* leaves collected in Brazil were air dried, ground, and combined as described to create a composite *P. marcgravii* sample. Likewise, *G. bilobum* was collected near Albany, Western Australia, Australia and subsequently air dried, ground, and combined to form a "MFA QC Pool" sample. Vegetative and/or floral material was sampled from herbarium specimens from the Missouri Botanical Garden of select Bignoniaceae and Malpighiaceae taxa: *F. elegans, N. stannea, N. multiglandulosa* (*Tetrapterys multiglandulosa*)*, N. acutifolia* (*T. acutiflolia*)*,* and *A.*

lasiandra (*Mascagnia lasiandra*). The *Tetrapterys* and *Mascagnia* species sampled have undergone a recent taxonomic revision accounting for the new scientific names.[33]

Extraction and derivatization reaction. Extraction of plant material for monofluoroacetate (MFA) was accomplished by the method of Lee et al.[8] Briefly, dry ground plant material (100 mg) was weighed into a 13 mL screw top test tube equipped with Teflon lined caps. Water (deionized, 5 mL) was added to each test tube and placed on a mechanical shaker for 30 min, then centrifuged (2570 x g; room temperature) for 5 min to separate the plant residue and water extract. The plant residue was extracted once more with water (5 mL) for 30 min, centrifuged, and the water extracts combined for a total of 10 mL. For herbarium specimens, ground plant material (25 mg) was weighed into a 2 mL screw-cap micro-centrifuge tube. Water (deionized, 1.5 mL) was added to each test tube and placed in a mechanical shaker for 18 h, then centrifuged to separate the plant residue and water extract. A water aliquot (1 mL) was filtered through a filter made from a Kimwipe (Kimberly-Clark Corp. Irving, TX) and a disposable pipette and subsequently transferred to a clean vial.

An aliquot (500 µL) of the water extract was transferred to a 5 mL screw top vial with a Teflon lined cap. The internal standard SMCA (50 µL of a 2.08 µg/mL solution) was added and the extract was then evaporated to dryness under a stream of N_2 at 40 °C. Concentrated sulphuric acid (30 µL) and *n*-propanol (70 µL) were added to the 5 mL screw top vial containing the evaporated extract reacted for 10 min. As the reaction was taking place, the vial was periodically stirred such that the sulfuric acid/*n*-propanol mixture came in contact with the surface of the vials where the residue of the evaporated water extract and SMCA solution remained. After the 10 min reaction time, water (2 mL) and chloroform (1 mL) were added to the 5 mL vial. The vial

158 was shaken vigorously and centrifuged (2570 x g; RT) for 5 min. The chloroform was removed
159 and filtered, through a filter made by inserting a section of Kimwipe (1 mm x 1 mm) into the top
160 of a disposable Pasteur pipette and pushing it to the top of the narrow portion of the pipette and
161 adding 0.35 g of anhydrous sodium sulfate, into a clean 5 mL screw top vial. The Kimwipe-
162 sodium sulfate filter was washed with chloroform (1 mL) into the 5 mL vial. The filtrate was
163 evaporated almost to dryness under stream of N_2 at room temperature, and reconstituted with
164 chloroform (200 μL).

165 To quantitate samples, a calibration curve with a range of 0.024 – 1.0 μg/mL (0.024, 0.039,
166 0.079, 0.16, 0.31, 0.47, 0.71 and 1.0 μg/mL) of MFA in water were prepared using SMFA and
167 SMCA prepared in the same manner as the samples. The concentrations of MFA in the
168 composite *P. marcgravii* extract were more concentrated than the other samples analyzed and
169 was diluted (625X) in water by two serial 1:25 dilutions. The standards/samples and the internal
170 standard were analyzed as propyl fluoroacetate (PFA) and the propyl chloroacetate (PCA),
171 respectively.

172 Spiked samples for recovery analysis were performed at two different levels in both the *A.*
173 *septentrionalis* composite and the *P. marcgravii* composite extracts. Ten μL and 20 μL of 2.04
174 μg/mL SMFA in water was spiked into four aliquots (0.5 mL) from extracts of 100 mg
175 *septentrionalis* composite and *P. marcgravii* composite extracts. This resulted in spikes at two
176 levels in *A. septentrionalis* composite (0.0157 μg MFA, n=4 and 0.0314 μg MFA, n=4) and *P.*
177 *marcgravii* composite (0.0157 μg MFA, n=4 and 0.0314 μg MFA n=3) extracts.

Instrumental conditions. GC-MS analyses were carried out using a 7890B gas chromatograph equipped with a split/splitless injector coupled to a 5977A MSD mass spectrometer and a 7693 autosampler (Agilent Technologies, Santa Clara, CA) which injected 1 μL of sample. Separations were performed using a 30 m x 0.250 mm i.d., DB-5MS column (Agilent Technologies, Santa Clara, CA). The carrier gas was helium at a flow of 1 mL/min. The oven temperature was 40 °C for 1 min; 40 - 60 °C at 3 °C/min; 60 °C - 250 °C at 30 °C/min; and at held 250 °C for 1 min for a total run time of 15 min. Split injection was performed with a 1:1 split ratio. The transfer line, ion source, and quadrupole temperatures were 275 °C, 230 °C, and 150 °C, respectively, the electron impact ionization voltage (EI) was 70 eV. The mass spectrometer was operated in both full scan and SIM modes with data acquired after a 4 min solvent delay. In full scan mode the mass range *m/z* 30-650 was scanned. In SIM mode *m/z* 43, 61, 79, and 91 were monitored from 4.0 – 6.4 min and *m/z* 49, 77, 79, 95, 97, 107 were monitored from 6.4 to 15.0 min. For quantitative analysis, ions with *m/z* 61, 79 and ions with m/z 49, 79, 97, 107 for PFA and PCA, respectively, were extracted and peak areas integrated. Under these conditions PFA eluted at 5.76 min and the PCA internal standard eluted at 9.34 min (Figures 1 and 2).

RESULTS AND DISCUSSION

Experimental rationale. Several analytical methods have been developed for the quantitation MFA in plants. Only one GC and/or GC-MS method has been reported for the quantitation of MFA in plant tissues.[16] This method required esterification of MFA with pentafluorobenzyl bromide and final analysis by GC-MS, but only after extensive sample extraction and preparation procedures including two, 72 h, pH-dependent liquid-liquid extraction steps to sufficiently

remove contaminants so that no interfering peaks were observed. The pH of the aqueous phase used in the liquid-liquid extractions had to be carefully monitored to prevent the sulfuric acid used in the extraction procedure from consuming the reaction reagent. Other reports have described the analysis of SMFA (1080) in water, blood, plasma, animal stomach contents, animal and human tissues by gas chromatography after esterification with methanol using diazomethane;[29] and by esterification with ethanol[30-32] and *n*-propanol[32] in the presence of concentrated sulfuric acid. The semi-quantitative method with the esterification of MFA by methanol and *n*-propanol reported by Peterson[32] required extensive sample preparation including the use of cation and anion exchange resins and distillation of fluoroacetate in the presence of concentrated sulfuric acid.[32] The methods described by Mori et al.[30] and Koryagina et al.[31] were simple in that the aqueous extract was evaporated to dryness and reacted with ethanol in the presence of sulfuric acid.[30, 31] However, in both these methods, the ethyl fluoroacetate was sampled using head space procedures for final GC analysis. In this study we investigated the reaction of short chained alcohols with MFA in the presence of sulfuric acid and were interested in developing a method in which an auto-sampler compatible with liquid sample solvents could be used.

Method development. We evaluated esterification of MFA and GC-MS analysis of the resulting MFA-esters with ethanol, *n*-propanol, and *n*-butanol. We were able to extract the resulting alkyl fluoroacetates from the aqueous reaction mixtures into chloroform for final GC-MS analysis. Detection of the butyl fluoroacetate derivative was problematic, with interfering peaks even after employing different oven temperature hold times and ramp rates. We also found that the ethyl fluoroacetate eluted on the tail of the solvent peak. However, the propyl

fluoroacetate (PFA) was resolved from interfering peaks and was retained sufficiently that it was well resolved from the solvent peak (Figures 1 and 3). This derivatization scheme was then directly applied to evaporated water extracts from MFA containing plants (Figures 1 and 5). The EI mass spectra of the PFA and the propyl chloroacetate (PCA) internal standard are shown in Figure 2.

Method validation. For quantitative purposes, ions at *m/z* 61, 79 for PFA and ions at *m/z* 49, 79, 97, 107 for PCA were extracted and peak areas integrated. A linear calibration curve (R^2=0.9944) over the range of 0.024 – 1.0 µg/mL µg/mL MFA in water with SMCA as an internal standard was used for quantitation. It should be noted that sodium fluoroacetate $^{13}C_2$ isotope is a superior choice for an internal standard and is commercially available, however, it was cost prohibitive for this application. The limit of detection (LOD, 3X S/N), and limit of quantitation (LOQ, 10X S/N) of MFA were determined to be 0.0079 µg/mL and 0.024 µg/mL, respectively (Figure 3). Additionally, recoveries of monofluoroacetate were determined at two different levels of MFA in both the *A. septentrionalis* composite and *P. marcgravii* composite samples. Recoveries of MFA ranged from 89.8-113% at both levels and in both of the different plants (Table 1). Intra-day and inter-day variation were also determined by the analysis the *A. septentrionalis* composite over 1 day (n=8) and seven days (n=7), respectively. The intra-day variation was determined to be 2.2% and the inter-day variation over 7 days was 4.6%.

Samples of *Gastrolobium* and *Amorimia* spp. were compared using different methods within and between laboratories. A *Gastrolobium bilobum* composite sample labeled “MFA QC Pool” was prepared and analyzed by scientists at the Department of Agriculture and Food, Western

Australia, South Perth, Western Australia (DAFWA) using the HPLC-MS method of Lee et al.[7] and by S. T. Lee at the Poisonous Plant Laboratory in Logan, Utah by the same method. (Table 2). This *G. bilobum* "MFA QC Pool" sample was analyzed by the GC-MS method described in this report. Comparison of the results in Table 2 between the HPLC-MS method run at laboratories in Australia and in the U.S.A. and with the GC-MS method reported herein are acceptable. Additionally, thirteen individual samples of *A. septentrionalis* plant parts consisting of stem, leaves, and floral plant parts previously analyzed using the HPLC-MS method described by Lee et al.[7] were also analyzed using the GC-MS method described. A good correlation described by the equation $y = 0.86x - 2.6$ ($R^2 = 0.993$) was observed between results using the HPLC-MS and GC-MS methods. This comparison supports the specificity and robustness of the developed method.

Detection of MFA herbarium samples. Herbarium specimens have been used previously to detect MFA in *Palicourea* and *Amorimia* species.[6,8] Using this method we analyzed herbarium specimens of several taxa reported or suspected to contain MFA. MFA was detected in all six of the *F. elegans* specimens analyzed using the GC-MS method in SIM mode. MFA was also detected in 8-25% of the *N. stannea, N. multiglandulosa, N. acutifolia,* and *A. lasiandra* specimens analyzed (Table 3). The detection frequency of MFA in these specimens was similar to those reported for *Amorimia* spp.[8] MFA was detected in *F. elegans* and *N. stannea*, consistent with the reports that these species cause sudden death.[1,4,9] In addition, MFA was detected in *N. multiglandulosa* and *N. acutifolia,* two species that are reported to cause sub-acute clinical signs similar to other MFA containing plants that cause sudden death.[4] Lastly, MFA was detected in *A. lasiandra*, a plant phylogenetically related to the *Niedenzuella* species investigated and not yet

reported to be toxic.[34] Caution should be taken with respect to the MFA concentrations reported from herbarium specimens. The herbarium concentration values reported may be biased due to a number of factors such as limited sample size, variability in the age of the herbarium specimens, and possible differences in the storage conditions etc.

In conclusion, an inexpensive, straight forward extraction, derivatization, sample preparation, GC-MS method for the analysis of MFA in plants was developed. The method compared favorably to a currently employed HPLC-MS method for the analysis of MFA in plants. This method was applied to the analysis of MFA in herbarium specimens and MFA was detected in *F. elegans, N. stannea, N. multiglandulosa, N. acutifolia*, and *Aenigmatanthera lasiandra*. This is the first report of MFA being detected in *F. elegans, N. multiglandulosa, N. acutifolia*, and *Aenigmatanthera lasiandra.* It is anticipated that the method is sufficiently simple for routine screening of suspected MFA-containing plants in Brazil and in other parts of the world where this toxin is a significant cause of livestock deaths.

SUPPORTING INFORMATION

List of plant taxa, voucher specimen numbers, and associated herbaria. This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE CAPTIONS

Figure 1. Reconstructed GC-MS ion chromatograms of (*m/z* 61.0, 79.0) from (A) water extract of *A. septentrionalis* composite sample and (B) water extract of *P. marcgravii* composite sample diluted 1:625 v:v. * denotes MFA peak (detected as propyl fluoroacetate). IS denotes internal standard peak.

Figure 2. Full scan mass spectrum of (A) propyl fluoracetate and (B) propyl chloroacetate. * denotes *m/z* used for qualitative determination of peak identity. ** denotes *m/z* used for quantitative analysis.

Figure 3. Reconstructed GC-MS ion chromatograms of (*m/z* 61.0, 79.0) from (A) a blank reaction (B) a 0.0079 µg/mL MFA standard (LOD) and (C) a 0.024 µg/mL MFA standard (LOQ). * denotes MFA peak as the propyl fluoroacetate derivative.

Table 1. Recoveries Obtained for Extracts of *A. septentrionalis* and *P. marcgravii* Spiked at Two Levels of MFA.

MFA Spike (µg)	0.0157		0.0314	
Samples	*A. septentrionalis* n = 4	*P. marcgravii* n = 4	*A. septentrionalis* n = 4	*P. marcgravii* n = 3
Mean Recovery (µg)	0.0141 ± 0.0005	0.0168 ± 0.0019	0.0324 ± 0.0023	0.0354 ± 0.0020
RSD (%)	3.54	11.3	7.10	5.65
Recovery (%)	89.8	107	103	113

Table 2. Comparison of Analysis of *G. bilobum* Composite MFA QC Pool Sample Between Different Laboratories and Different Analysis Methods.

Laboratory	Method	MFA ± STDV (μg/g)
DAFWA n = 10	HPLC-MS	50 ± 5
PPRL n = 4	HPLC-MS	60 ± 3
PPRL n = 4	GC/MS	47 ± 2

Table 3. GC-MS Analysis of Herbarium Specimens Representing Different Plant Taxa Reported or Suspected of Containing MFA.

Family	Scientific Name	Number of Specimens	Specimens Containing MFA	MFA Conc. Range (μg/g)
Bignoniaceae	*Fridericia elegans*	6	6	0.31 – 1.3
Malpighiaceae	*Aenigmatanthera lasiandra*	8	2	nd – 5.2
Malpighiaceae	*Niedenzuella acutifolia*	8	1	nd – 0.21
Malpighiaceae	*Niedenzuella multiglandulosa*	8	1	nd – 1.7
Malpighiaceae	*Niedenzuella stannea*	13	1	nd – 1.4

Figure 1

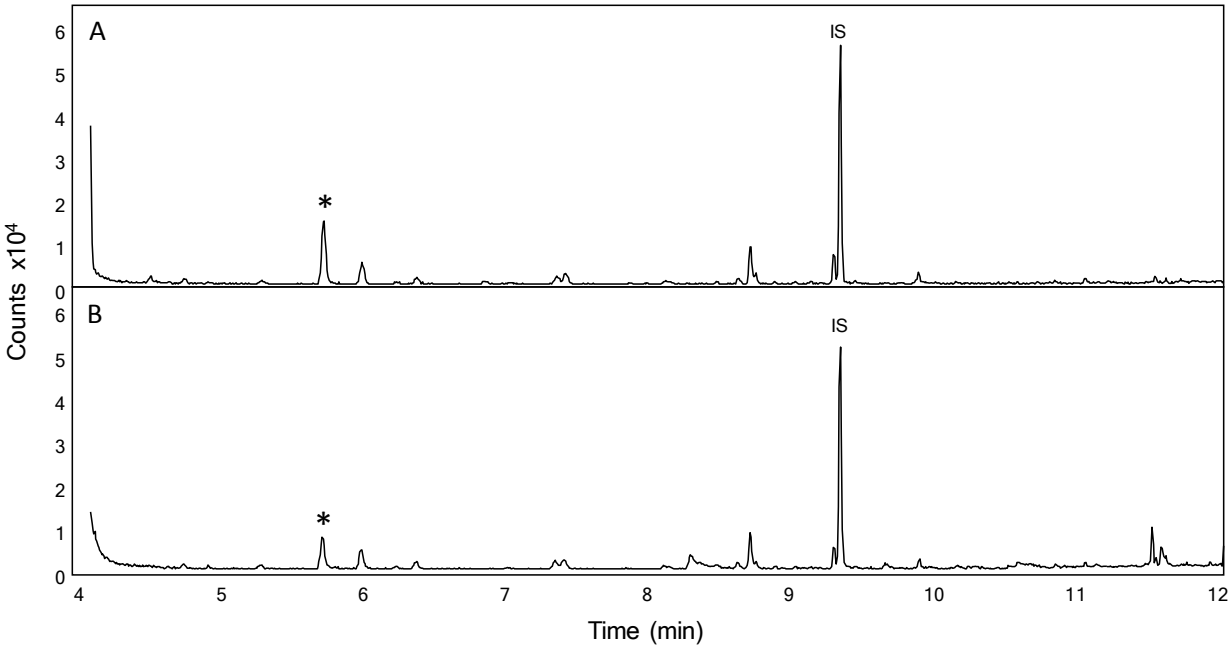
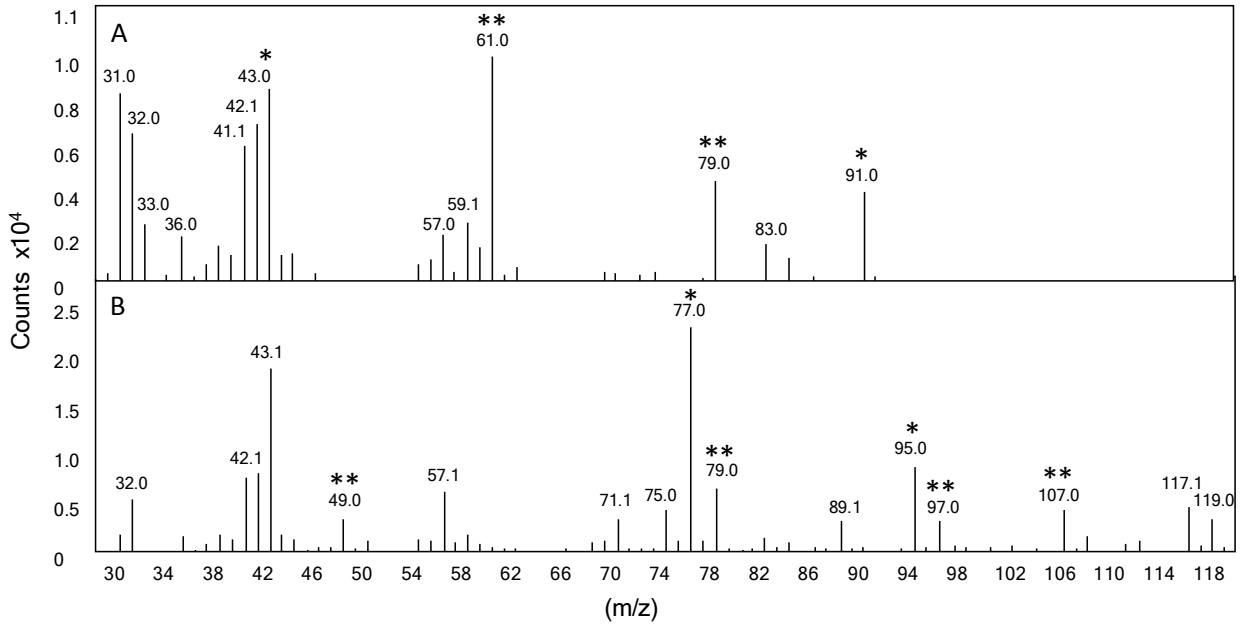


Figure 2


A
B
Counts x10^4
(m/z)
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32.0
33.0
36.0
41.1
42.1
43.0
57.0
59.1
61.0
79.0
83.0
91.0
32.0
42.1
43.1
49.0
57.1
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77.0
79.0
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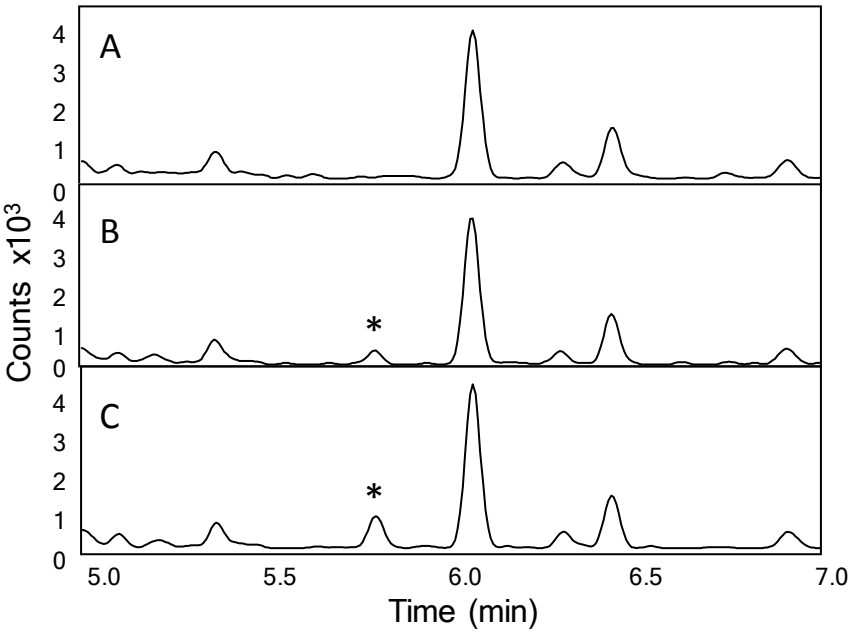
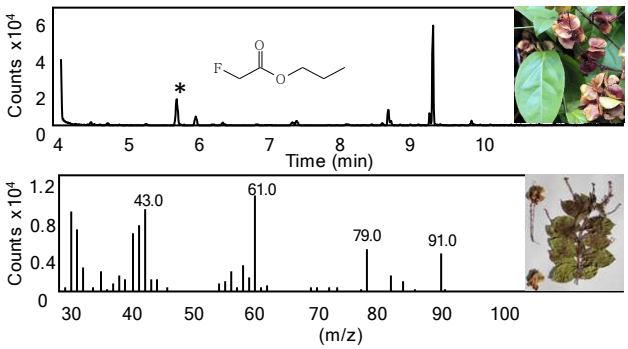
95.0
97.0
107.0
117.1
119.0

Figure 3



TOC Graphic


Counts x10^4
6
4
2
0
4
5
6
7
8
9
10
Time (min)
F
O
O
*
Counts x10^4
1.2
0.8
0.4
0
43.0
61.0
79.0
91.0
30
40
50
60
70
80
90
100
(m/z)