

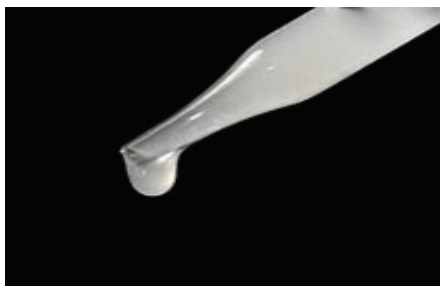
# Analysis of Emu Oils Using Ultra Performance Liquid Chromatography—Time-of-Flight Mass Spectrometry

LECO Corporation; Saint Joseph, Michigan USA

**Key Words:** Accurate Mass, Emu Oil, Fragmentation, High Resolution Time-of-Flight MS and MSc<sup>2</sup>, Metabolites Databases, UHPLC separation

## 1. Introduction

Emu oil is made from the fat of the emu, a bird native to Australia. Emu oil has been traditionally used by the Australian aborigines as a non-traditional medicine.<sup>[1]</sup> Modern medical research shows that Emu oil and its modified forms have unique health benefits. Emu oil is marketed and promoted as a great natural product with a wide variety of claimed health benefits.<sup>[2]</sup>



Emu oil is composed of approximately 70% unsaturated fatty acids, but more rigorous molecular characterization is not readily available. Specifically, the key components responsible for its therapeutic properties are not well known. Global regulatory agencies are working to establish modern control methods for non-traditional therapeutics and mass spectrometry is a logical analytical tool owing to its inherent comprehensive nature. High performance time of flight mass spectrometry offers the most comprehensive capabilities.

In this study we provide preliminary characterization of emu oils of different “potency” with the ultimate objective of correlating composition and activity. UHPLC is coupled to the Citius™ LC-HRT, a novel high performance platform employing Folded Flight Path™ (FFP™) technology. Fragment ion spectra are generated using MSc<sup>2</sup> technology and public access metabolite databases are utilized to confirm the analytes identification.<sup>[3] [4]</sup>

## 2. Results and Discussions

The LC-MS total ion chromatograms (TIC) for the five Emu oil samples are provided in Figure 1. The infusion of Agilent ESI Tuning Mix occurs from 1650 to 1680 seconds in each analysis for mass re-calibration.

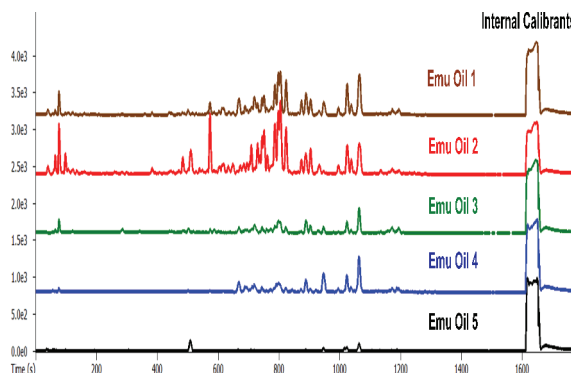


Figure 1. TIC for the 5 Methanolic Emu Oil Extracts.

Automated processing of positive and negative ion data provided a large and varied number of analytes for each sample with parent and fragment ion information including formula identification. The numbers of analytes in each sample are summarized in Table 1. These processed data were further evaluated for structural information by input into public metabolite databases (METLIN and KEGG) to facilitate analyte identification.

Table 1. Numbers of analytes detected in positive and negative ESI modes by UHPLC-TOFMS.

Emu Oil Sample	Positive Ions	Negative Ions
1	407	911
2	494	1269
3	345	1288
4	251	589
5	114	231

Table 2 shows the ten most abundant ions in Emu oil sample 1 along with their proposed formula.

Table 2. Characteristics of the 10 most abundant positive ions of Emu Oil sample 1.

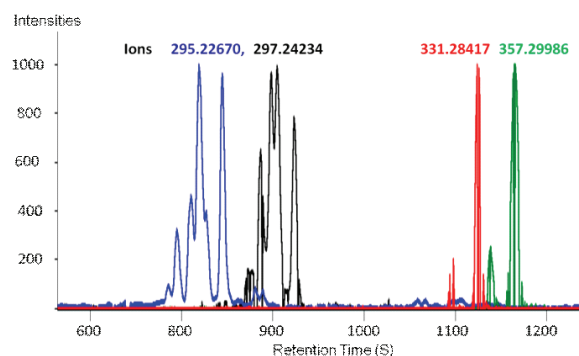
R.T. (s)	Base Mass	Formula
786.976	297.24235	C18H32O3
803.696	297.24234	C18H32O3
720.088	295.22672	C18H30O3
1065.371	357.29986	C21H40O4
1024.924	331.28417	C19H38O4
823.821	297.24238	C18H32O3
775.042	332.27943	C18H37O4N
751.75	561.48767	C36H64O4
1063.94	379.28172	C19H34O2N6
889.637	299.25792	C18H34O3

Input of all ten of these base mass values into the METLIN search engine resulted in 239 total hits. Nine of ten ions showed at least one positive identification with mass accuracy less than 1 ppm, where the other three ions had mass accuracies less than 7 ppm as shown in Table 3. Amongst the matches, isomeric species were frequent and cannot be distinguished using MS alone.

Additional information on the nature of the analyte can be achieved using fragment ion analysis and this is further empowered having the fragment ions at high resolution and high mass accuracy. The Citius offers this capability in the form of MSc<sup>2</sup>.

**Table 3. Highest-probability Search Results of positive ions of Emu Oil 1.**

Input Mass	Δppm	Name	Formula
297.24235	0.20	&alpha;-artemismic acid	C18H32O3
299.25792	0.60	Ricinoleic acid	C18H34O3
299.25792	0.60	Ricinelaic acid	C18H34O3
297.24238	0.13	Dimorphecolic acid	C18H32O3
331.28417	0.36	MG(0:0/16:0/0:0)	C19H38O4
357.29986	0.22	MG(0:0/18:1(9Z)/0:0)	C21H40O4
379.28172	6.80	MG(0:0/20:4(8Z,11Z,14Z,17	C23H38O4
561.48767	0.11	Mayolene-18	C36H64O4



**Figure 2. Extracted ion chromatograms at m/z 297.24234, 295.22670, 331.28417 and 357.29986 from representative emu oil using a 0.001 Da window.**

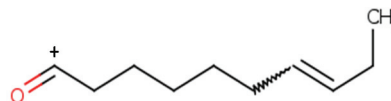
The protonated ion (M+H)<sup>+</sup> observed at 297.24234 was confirmed by observing additional adduct and dimer species from this molecule in the spectrum such as (M+Na)<sup>+</sup>, (M+K)<sup>+</sup>, (M+H-H<sub>2</sub>O)<sup>+</sup>, (M+H-2H<sub>2</sub>O)<sup>+</sup>, (2M+H)<sup>+</sup>, (2M+Na)<sup>+</sup>. Formula assignment using accurate mass analysis provides the opportunity for repetitive measurement. This is shown in Table 4 and provides nine measurements of the analyte of interest each of which occurred with an error of less than 0.7 ppm.

**Table 4. Mass 297.24234 and its associated adduct ions**

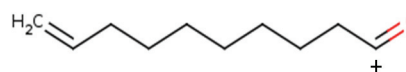
Ions	Species	Formula	Mass	Δ PPM
297.2423	(M+H) <sup>+</sup>	C18H33O3	297.24242	-0.2691
279.2319	(M+H-H <sub>2</sub> O) <sup>+</sup>	C18H31O2	279.23185	0.0000
261.2212	(M+H-2H <sub>2</sub> O) <sup>+</sup>	C18H29O1	261.22129	-0.3828
233.2264	(M+H-CO-2H <sub>2</sub> O) <sup>+</sup>	C17H29	233.22638	0.2864
314.2691	(M+NH <sub>4</sub> ) <sup>+</sup>	C18H36O3N	314.26897	0.2864
319.2245	(M+Na) <sup>+</sup>	C18H32O3Na	319.22437	0.3759
335.1985	(M+K) <sup>+</sup>	C18H32O3K	335.1983	0.4475
360.2511	(M+MeCN+Na) <sup>+</sup>	C20H35O3NaN	360.25092	0.6107
593.4776	(2M+H) <sup>+</sup>	C36H65O6	593.47757	0.0674

In the high energy channel spectrum of m/z 297.24234 a fragment ion at m/z 153.12739 was observed and can be assigned to a formula of C<sub>10</sub>H<sub>17</sub>O with 65 ppb mass accuracy (structures A and B below). This formula is consistent with fragment ions obtained from unsaturated fatty acids based on a fragment search in the METLIN data base.

A.



B.



A closer evaluation of the extracted ion chromatogram from m/z 297.24234 (Figure 2) suggests other analytes which may be closely related or isomeric.

In a similar fashion, other m/z values detected in the emu oils were searched against the METLIN database. A representative set of m/z values and the associated number of hits are provided in Table 5 for positive ion and Table 6 for negative ion analyses. This includes ONLY hits having a mass error of <1 ppm. Accurate mass fragment ions, which are deconvoluted from the complex signal, provide a substantial reduction in the number of candidates.

**Table 5. Spectra Matches by (+)ESI TOF MS and MSc<sup>2</sup>.**

Analytes (m/z)	Matches for Molecular Ions	Fragment Ions	MS/MS Match with 10 CID Ions
297.24235	41	100	6
297.24234	41	100	6
295.22672	40	100	5
357.29986	17	100	7
331.28417	48	100	6
297.24238	41	100	6
332.27943	12	100	5
561.48767	3	100	0
379.28172	7	100	1

**Table 6. Identifications by MS and high energy MSc<sup>2</sup> Spectra Matches by (-)ESI TOF MS and MSc<sup>2</sup>.**

Analytes (m/z)	Hits for Molecular Ions	Fragment Ions	MS/MS Match with 10 CID Ions
253.2193	37	100	0
255.23512	22	100	0
313.24033	40	100	0
297.24607	53	100	0
295.23048	36	100	10
279.23505	144	100	2
187.09849	31	100	2
297.24559	71	100	2
315.25706	39	100	0
299.26149	30	100	0

### 3. Conclusions

Analysis of highly complex lipid samples using high performance TOFMS and MS<sup>2</sup> on the Citius LC-HRT using UHPLC for analyte introduction and separation has provided excellent qualitative data for both molecular (precursor) and product ions. This has provided rich information to facilitate the identification of active components in different oils. High resolution data were acquired with MS<sup>2</sup> experimentation in both positive and negative ESI ionization modes. Accurate mass fragment ion analysis in combination with database searches permitted substantial reduction in the number of potential identities for the analytes. These data demonstrate the utility of accurate mass TOFMS as achieved on the Citius LC-HRT instrument in characterizing complex lipids and providing accurate mass fragment ions for database-assisted identification.

### 4. References

- 1) Jenni Harrold and Joanne Whitby (1996). Aboriginal studies: Developing an awareness of Aboriginal people and their culture. R.I.C. Publications. p. 16. ISBN 978186.
- 2) Yoganathan S, Nicolosi R, Wilson T, *et al.* (2003). Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. *Lipids* **38** (6): 603–7.
- 3) Smith C.A., O'Maille G., Want E.J., Qin C., Trauger S.A., Brandon T.R., Custodio D.E., Abagyan R., Siuzdak G. METLIN: a metabolite mass spectral database. *Therapeutic Drug Monitoring*, 2005, 27(6), 747-751.
- 4) C.A. Smith, E.J. Want, G.C. Tong, R. Abagyan, and G. Siuzdak. XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. *Analytical Chemistry*, 2006, 78(3), 779-787.

### 5. Sample Preparations

Five Emu oil samples (Meon Laboratories, Australia) were heated at 60 °C for ten minutes until yellow clear solutions were formed. A 500 µl aliquot of each sample was mixed with 500 µl of HPLC grade methanol and incubated at 4°C overnight then centrifuged at 10,000 rpm for 10 min. A portion of the top methanol layer from each sample was collected and diluted ten-fold with HPLC grade methanol (20 µl to 200 µl) for analysis.

### 6. Experimental Conditions

#### Mass Spectrometry

Data were acquired in both positive and negative ion modes using the electrospray interface on the LECO Citius LC-HRT in high-resolution (HR) mode (R(FWHM) = 50,000). Detailed acquisition parameters are summarized below.

Ion polarity	Positive and Negative
Nozzle Temperature (°C)	100
Desolvation heater temp (°C)	900
Desolvation flow (s/m)	7.5
Nebulizer pressure (PSI)	50
Spectral acquisition rate	2.5 spectra / second
CID Mode	Pulsed (60 eV)
Spectral range (m/z)	50 to 1000

#### UHPLC Conditions

A Shimadzu UFPLC was used with samples maintained at 5°C. Waters 1.8 µm HSS T3 C18 column (2.1 x 100 mm) was maintained at 40° C and separation was achieved using 0.1% formic acid (FA) in HPLC grade water (A) and acetonitrile (B). The gradient was 40-100%B over 20 minutes.

