SUPPLEMENTARY MATERIAL

Molecular profiling of Peru Balsam reveals active ingredients responsible for its pharmaceutical properties

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Peru Balsam, a resinous substance derived from *Myroxylon balsamum* var. *pereirae*, has historically been used as a topical ointment for various skin conditions such as scabies, poorly healing wounds, eczema, and haemorrhoids. The ingredients responsible for these properties are not fully elucidated. We investigated the chemical composition of two Peru Balsam samples, one historical and one modern, using gas chromatography/mass spectrometry to identify the active ingredients responsible for its pharmaceutical properties. Both Peru Balsam specimens investigated had similar compositions, showing the stability of the substance. Components identified are effective against scabies, exhibit antimicrobial activity and aid skin penetration. These properties are consistent with historical uses of Peru Balsam. Several ingredients are also known allergens. This study, combining chemical information with scientific literature related to pharmaceutical properties of natural substances, represents a breakthrough in the elucidation of active ingredients in Peru Balsam.

Keywords: Peru Balsam; Myroxylon balsamum; Meso- and Southern America; gas chromatography/mass spectrometry; allergy; antimicrobial

Materials and Methods

Chemicals and Standards

Dichloromethane and methanol (HPLC grade, Sigma-Aldrich, USA) were used for sample dissolution. Derivatisation was performed using N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) with 1% w/w trimethylsilyl chloride (Sigma-Aldrich, USA). Tridecanoic acid (Sigma-Aldrich, USA) was used as internal standard for the derivatisation procedure. 2,2,4-trimethylpentane (Pesticide grade, Honeywell Riedel-de-Haen, USA) was used as injection solvent.

Specimen of Peru Balsam

The historical sample was provided by the Royal Pharmaceutical Society Museum (Reference No. LDRPS:2004.44.28). The medicinal preparation has been part of the collection since 2004 (Fig. S1). The rear dispensing label states it contains 25 g of Balsam of Peru. It was manufactured by the firm John Bell, Hills and Lucas Limited. This Peru Balsam bottle was then dispensed in a pharmacy in 1963. The bottle's content is viscous, of a dark brown colour and has a sweet smell similar to vanilla. Based on the 'actions and uses' section in the 1963 British Pharmaceutical Codex (BPC), "Peru Balsam has been used as an ointment (12.5 per cent v/v) for the treatment of scabies, but for this purpose it has been superseded by benzyl benzoate application. It is an ingredient of compound bismuth subgallate suppositories, used for the symptomatic relief of haemorrhoids. Diluted with an equal part of castor oil, the balsam has been used as an application to bed-sores and chronic ulcers. Continued application of the balsam to the skin may cause sensitisation." (Pharmaceutical Society of Great Britain, 1963).

The modern sample of Peru Balsam (CAS number: 8007-00-9) was purchased from G. Baldwin & Co. The sample originated in El Salvador and was obtained by scorching or inflicting V-shaped wounds on the bark of the trunk of the tree *Myroxylon balsamum* var. *pereirae*. The liquid was collected by placing cloth around the trunks. These cloths were then boiled in water to separate the resin from the cloth and the water was boiled off to leave Peru Balsam.

Figure S1. Illustration of the *Myroxylon balsamum* var. *pereirae* plant (Köhler 1897).

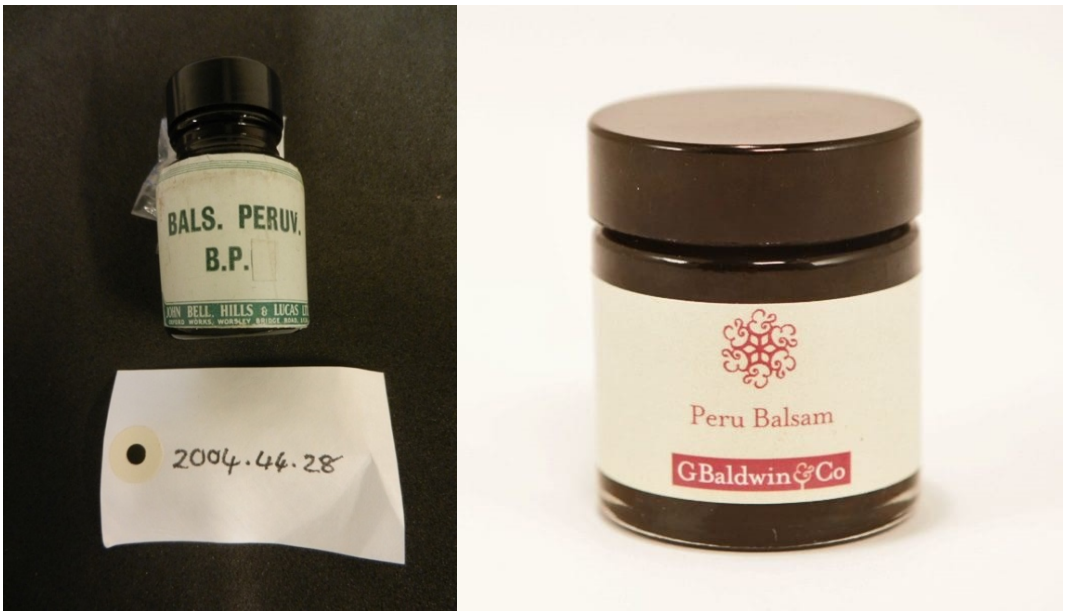

Figure S2. (a) Bottle containing Peru Balsam preserved at the Royal Pharmaceutical Society Museum (Reference No. LDRPS:2004.44.28); (b) Modern sample of Peru Balsam purchased from G. Baldwin & Co.

GC/MS Analysis

A 1 mg sub-sample of each Peru Balsam specimens was dissolved in 1 mL of 1:1 (v/v) dichloromethane:methanol mixture. To achieve complete dissolution, samples were also sonicated for 10 min at 40 °C, and clear solutions were obtained. A 20 µL aliquot of the solution was dried under a nitrogen stream and then derivatised with 20 µL of bis-trimethylsilyl-trifluoracetamide (with 1% w/w trimethylsilyl-chloride) at 60°C for 30 min in a water bath and dissolved in 150 µL 2,2,4-trimethylpentane, Finally, 2 µL were injected in the GC/MS system.

GC/MS analyses were performed with a 6890 gas chromatograph equipped with a PTV (Programmable Temperature Vaporizing) injector and coupled with a 5975 Mass Selective Detector (Agilent Technologies, USA). Separation was achieved using an HP-5MS capillary column (30 m x 250 µm, film thickness 0.25 µm) and helium as carrier gas at a constant flow of 1.2 mL/min. Injection was performed in splitless mode at a temperature of 280 °C. Injection volume was 2 µL. The following temperature program was used for the GC oven: 80 °C isothermal for 2 min; 10 °C/min up to 200 °C, then isothermal for 1 min; 10 °C/min up to 280 °C, then isothermal for 50 min. The transfer line to the MS was kept at 280 °C. The mass spectrometer was operated in EI positive mode (70 eV, m/z range 50-800). The ion source and quadrupole analyser were kept at 230 °C and 150 °C, respectively.

Compounds were identified based on their mass spectra, by comparison with reference libraries (Wiley and NIST-EPA-NIH) and with published literature.

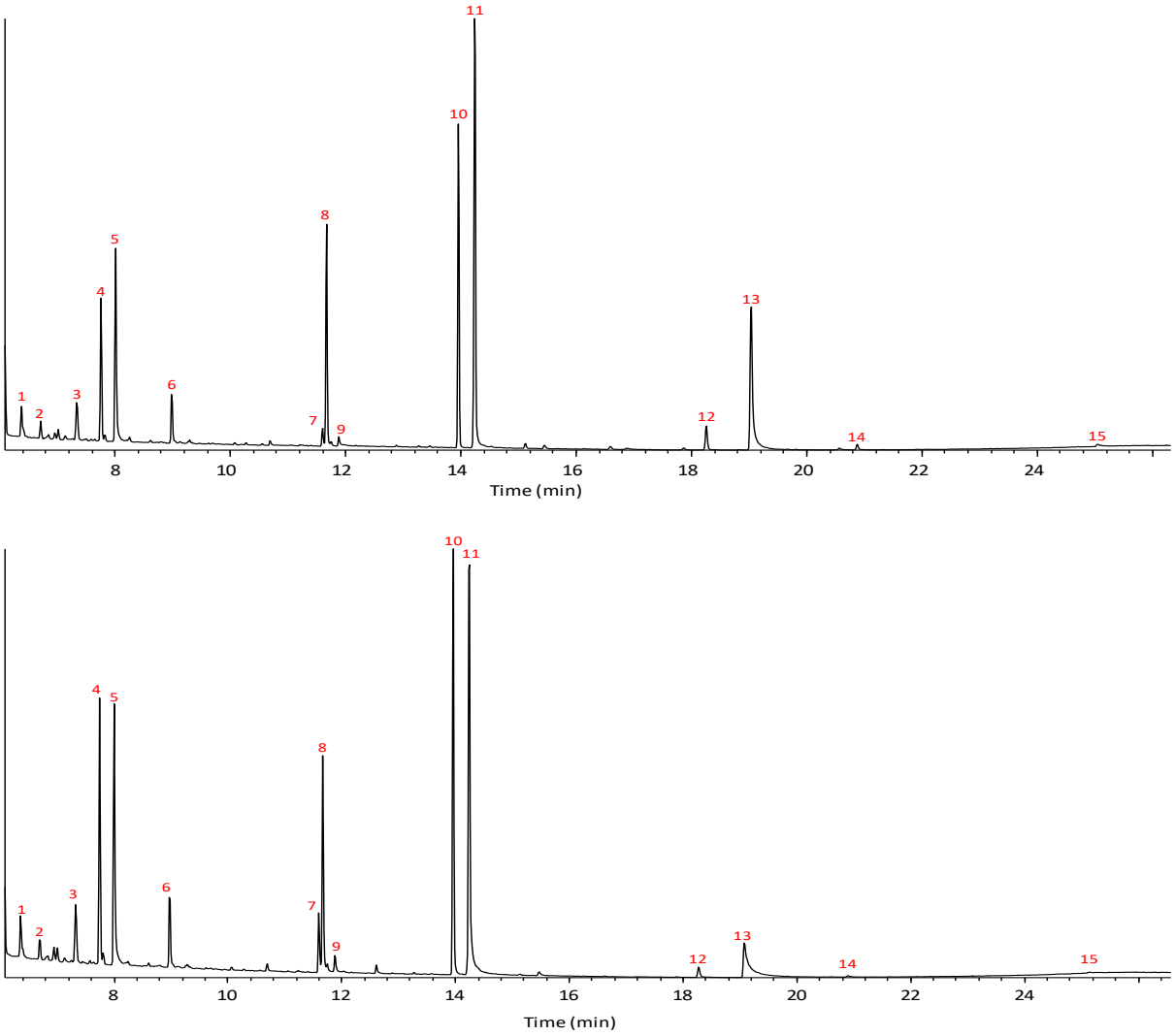

Figure S3. Total Ion Chromatograms (TIC) obtained on the modern reference sample (a) and the historical sample (b) of Peru Balsam. (#1) 3,3-dihydroxyprop-2-enal (2 TMS); (#2) unknown compound 1; (#3) unknown compound 2; (#4) benzoic acid (TMS); (#5) 3,4-dimethoxycinnamaldehyde; (#6) unknown compound 3; (#7) vanillin (TMS); (#8) cinnamic acid (TMS); (#9) nerolidol; (#10) tridecanoic acid (TMS, IS); (#11) benzyl benzoate; (#12) palmitic acid (TMS); (#13) benzyl cinnamate; (#14) stearic acid (TMS); (#15) coniferyl benzoate (TMS). A list of the identified compounds and their main *m/z* signals is reported in Table S1.

Table S1. Principal markers determined by GC/MS in the two samples of Peru Balsam analysed in this study. Retention time, name and MS fragmentation data are provided for each molecule identified (mass spectra in supplementary information). TMS indicates that the molecules are derivatised. IS corresponds to internal standard.

#	t_R (min)	Name	*m/z*
1	6.4	3,3-dihydroxyprop-2-enal (2 TMS)	232, 204, 191, 147, 73
2	6.7	unknown compound 1	217, 202, 189, 174, 146, 130, 73
3	7.3	unknown compound 2	217, 202, 189, 174, 146, 130, 73
4	7.8	benzoic acid (TMS)	194, 179, 135, 105, 77, 51
5	8.0	3,4-dimethoxycinnamaldehyde	192, 177, 163, 123
6	9.0	unknown compound 3	298, 283, 267, 229, 197, 173, 156, 130, 89
7	11.6	vanillin (TMS)	224, 209, 194, 75, 57
8	11.7	cinnamic acid (TMS)	220, 205, 161, 145, 131, 103, 75
9	11.9	nerolidol	204, 189, 161, 136, 123, 107, 93, 69, 55, 41
10	14.0	tridecanoic acid (TMS, IS)	286, 271, 145, 132, 129, 117, 75, 73
11	14.2	benzyl benzoate	212, 194, 167, 105, 91, 77, 65
12	18.3	palmitic acid (TMS)	328, 313, 145, 132, 129, 117, 75, 73
13	19.1	benzyl cinnamate	238, 220, 192, 178, 147, 131, 115, 103, 91, 77, 65, 51
14	20.9	stearic acid (TMS)	356, 341, 145, 132, 129, 117, 75, 73
15	25.1	coniferyl benzoate (TMS)	356, 341, 326, 249, 222, 192, 91, 73

Variation of the proportions of benzyl esters

The main difference between the historical sample and the modern reference one is the relative intensities of the peaks of benzyl esters and their corresponding acids. To highlight this difference, the peaks of benzyl-benzoate (#11), benzoic acid (#4), benzyl-cinnamate (#13) and cinnamic acid (#8) were integrated and area ratios were calculated (Tab. S2). Both acid/ester ratios were higher for the historical sample, suggesting that this sample underwent partial hydrolysis. No peak was found that could be attributed to benzyl alcohol, even in the historical sample in which the amount of free acids is higher. This may be due to autoxidation reaction of alcohol to give the corresponding aldehyde and carboxylic acid, which has been reported for both benzyl alcohol and cinnamyl alcohol (Sudareva and Chubarova 2006; Niklasson et al. 2013). Some of the difference may also be due to variation of the plant

material source. However, given the volatility and instability of the main constituents and the large age difference between the samples, at least some of the differences in composition are likely due to degradation processes (Courel et al. 2019).

Table S2. Peak areas of benzoic acid, benzyl-benzoate, cinnamic acid and benzyl-cinnamate and the ratios benzoic acid / benzyl-benzoate and cinnamic acid / benzyl-cinnamate showing a hydrolysis of the esters for the historical sample due to natural degradation.

	Reference sample	**Historical sample**
benzoic acid	7068327	8286185
benzyl-benzoate	26822707	19838060
cinnamic acid	10277638	6933499
benzyl-cinnamate	14554145	5301234
benzoic acid / benzyl-benzoate	0.26	0.42
cinnamic acid / benzyl-cinnamate	0.71	1.31

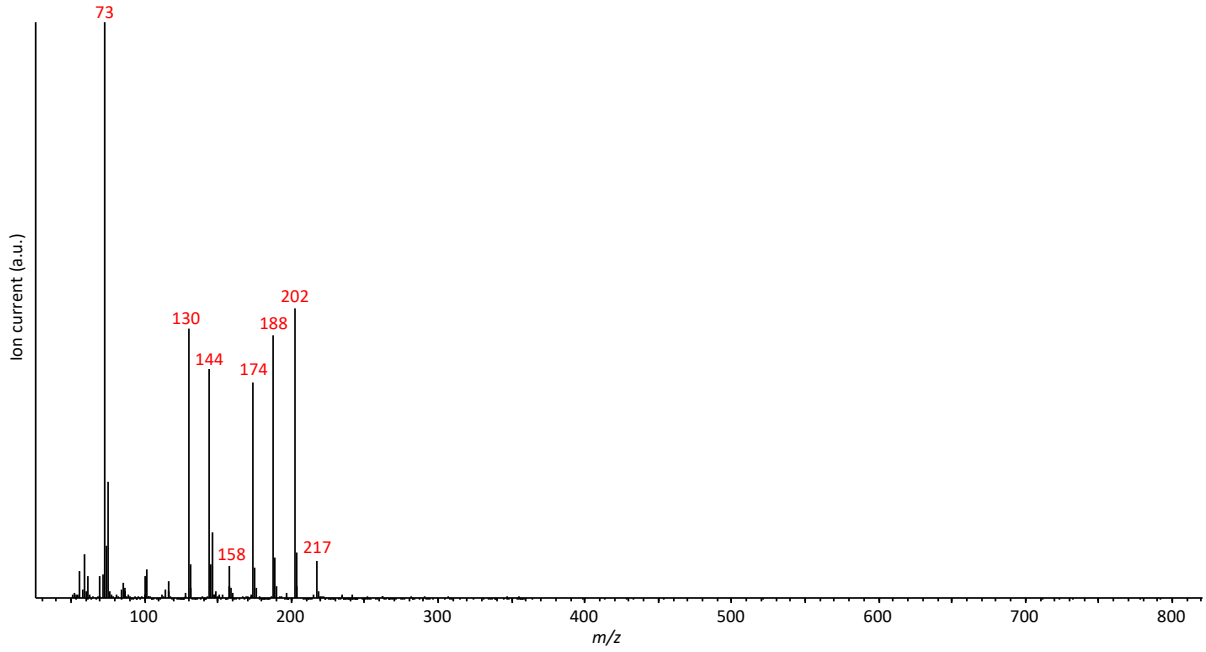

Figure S4. Mass spectrum of compound #2.

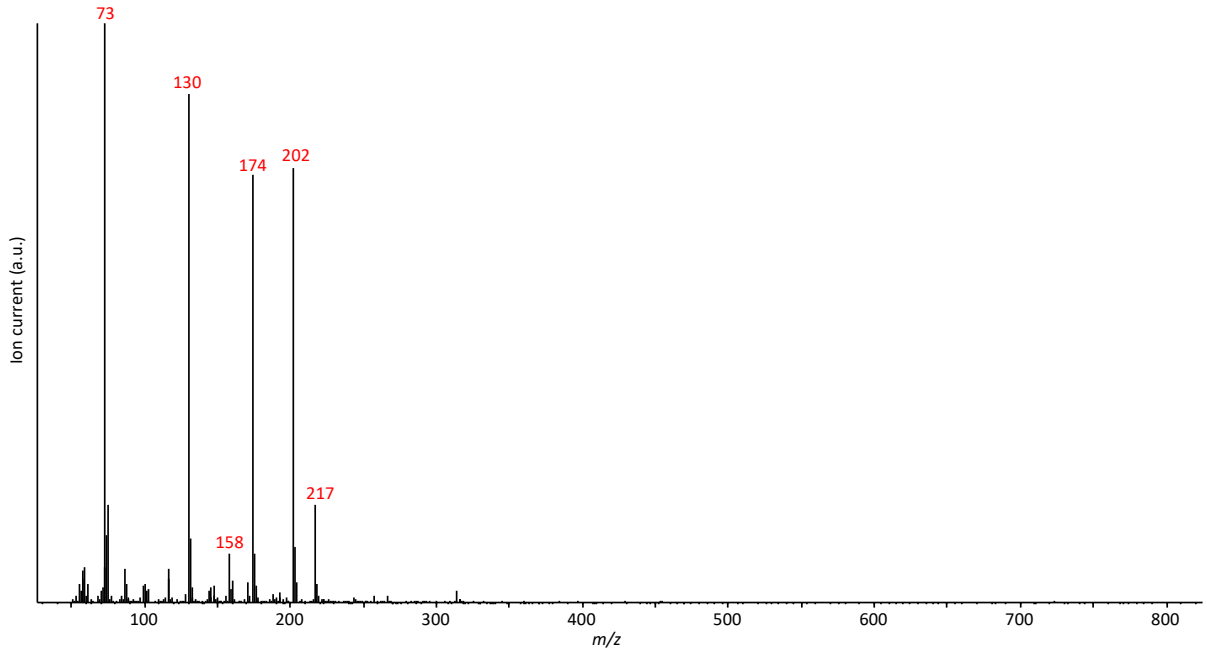

Figure S5. Mass spectrum of compound #3.

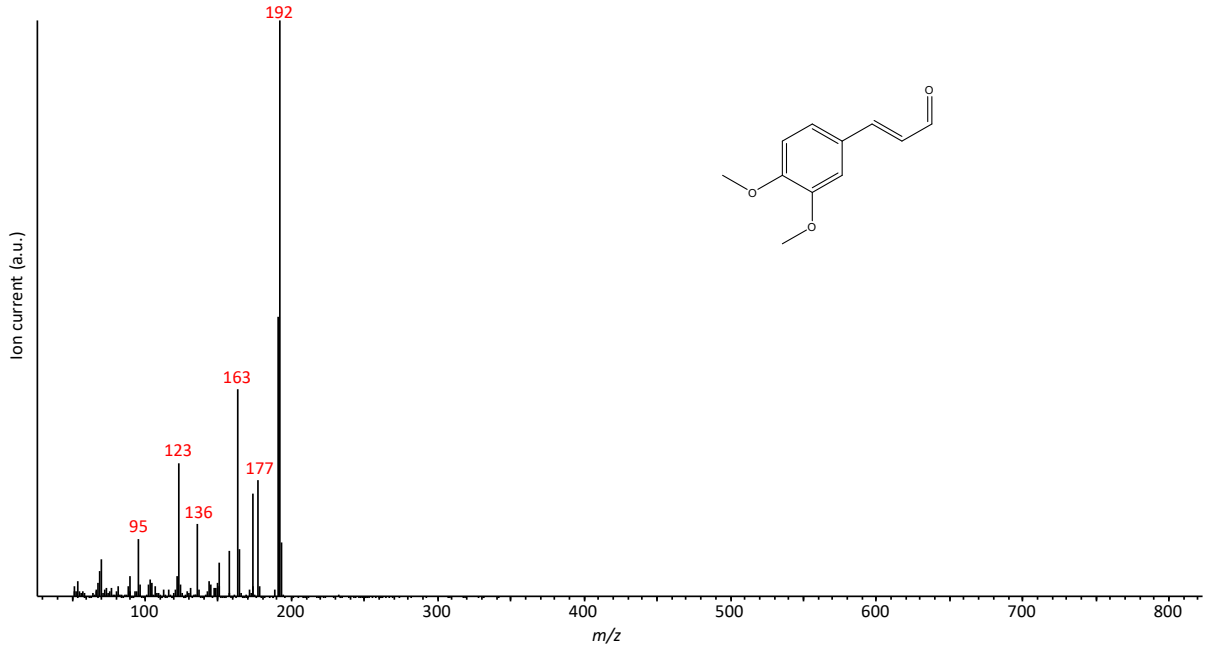

Figure S6. Mass spectrum and formula of 3,4-dimethoxycinnamaldehyde - Compound #5.

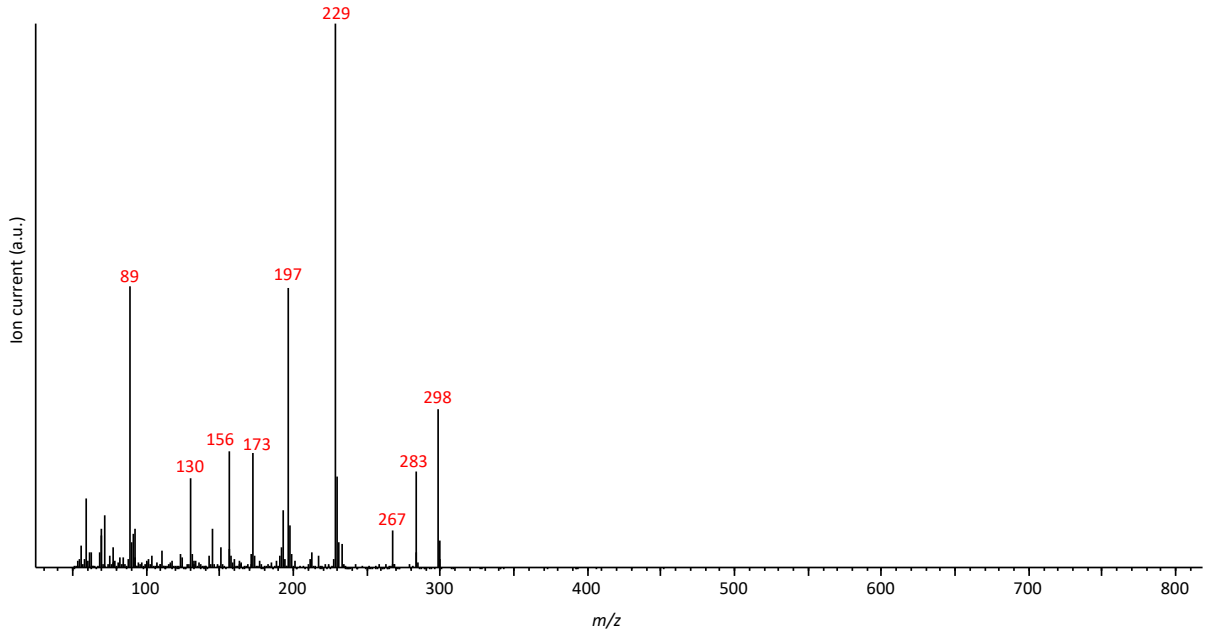

Figure S7. Mass spectrum of compound #6.

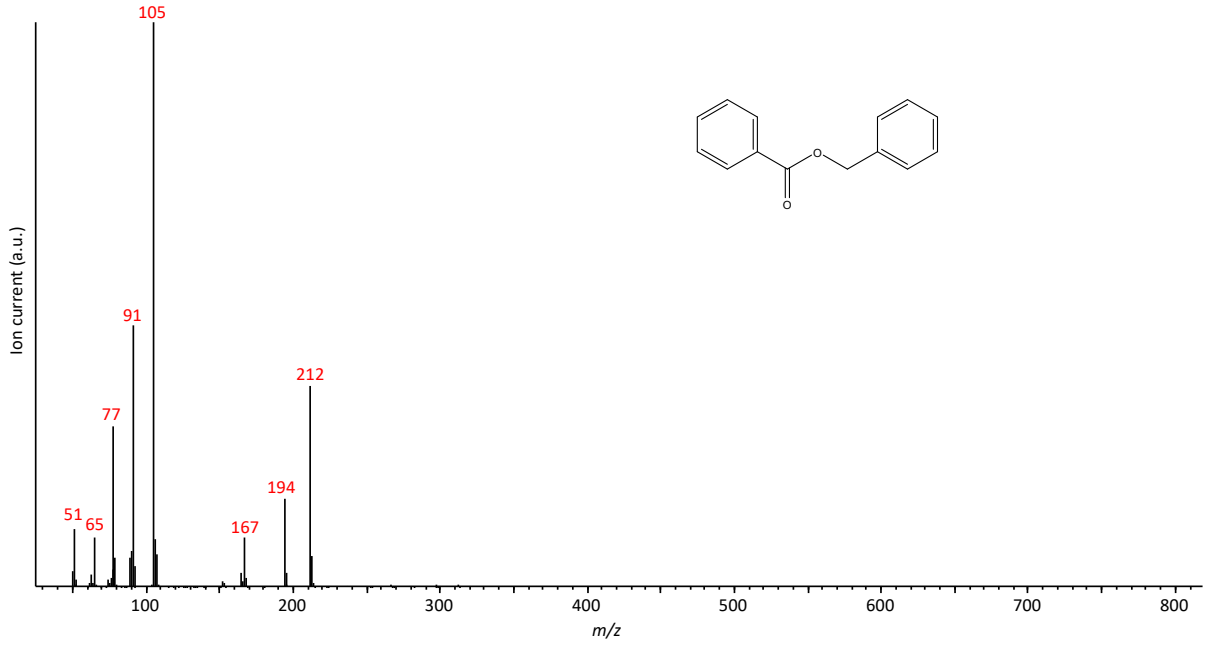

Figure S8. Mass spectrum and formula of benzyl benzoate – Compound #11.

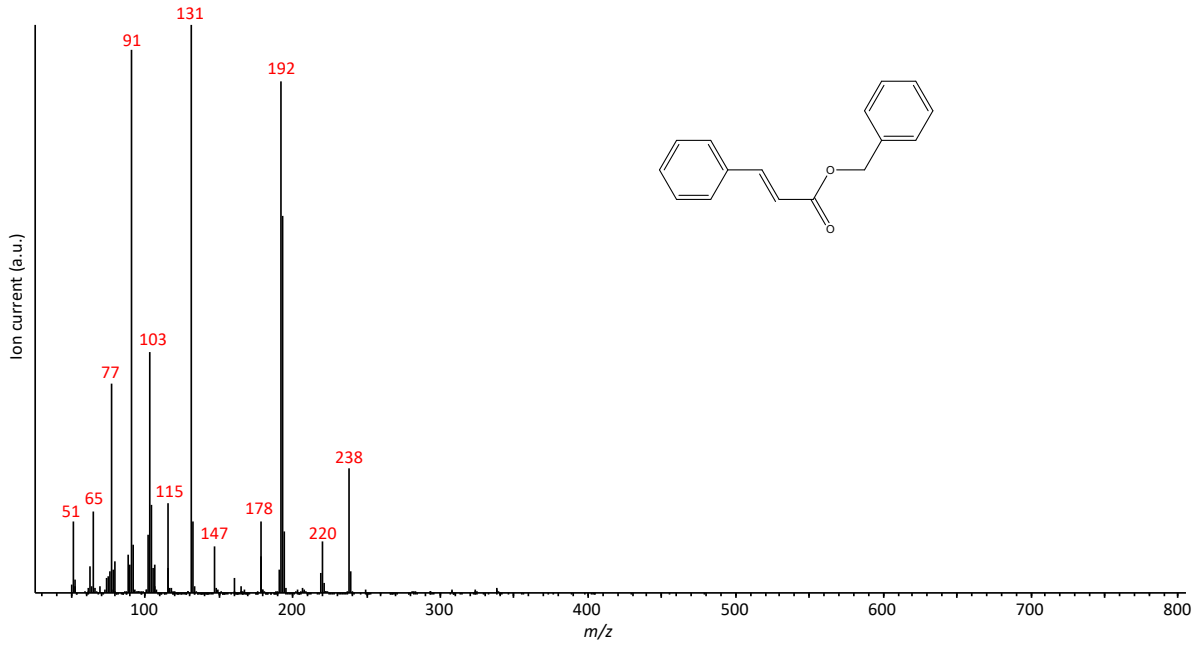

Figure S9. Mass spectrum and formula of benzyl cinnamate - Compound #13.

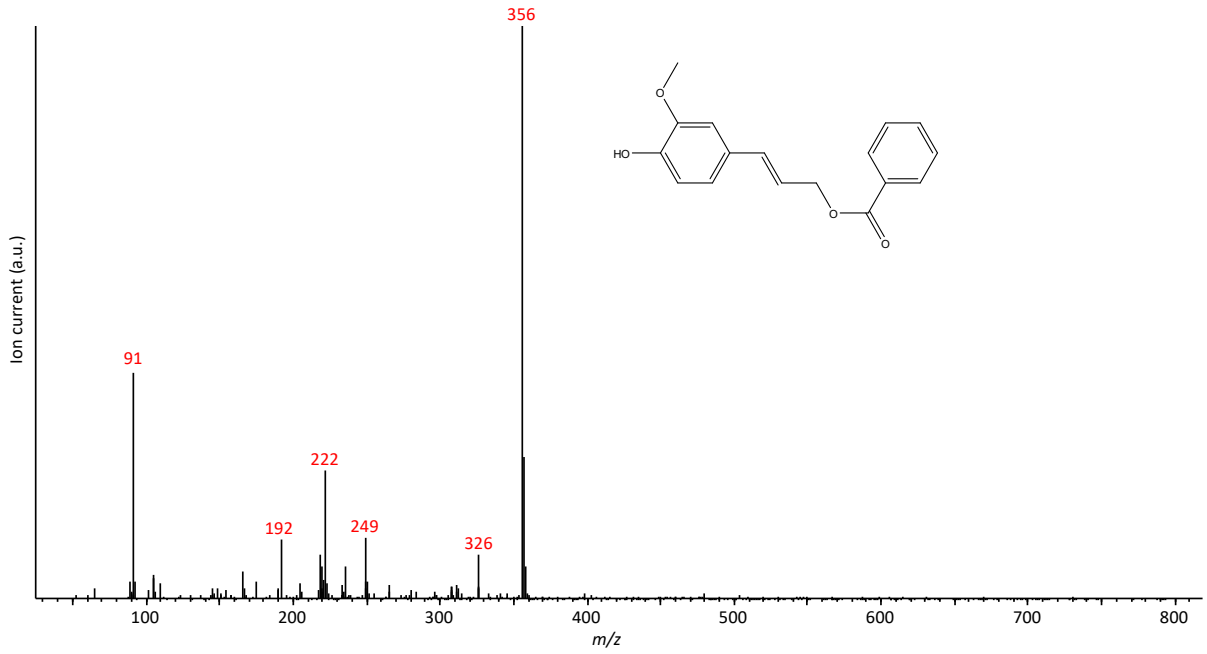

Figure S10. Mass spectrum and formula of coniferyl benzoate - Compound #15.

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