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Identification of atranorin and related potential allergens in oakmoss absolute by high-performance liquid chromatography–tandem mass spectrometry using negative ion atmospheric pressure chemical ionization

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Abstract

This paper describes the first high-performance liquid chromatographic–tandem mass spectrometric method for the identification of atranorin and related potential allergens in oakmoss absolute. Oakmoss absolute is ubiquitous in the fragrance industry and is a key component in many fine perfumes. However, oakmoss absolute causes an allergic response in some individuals. Research is focused toward establishing the identity of the compounds causing the allergic response so a quality controlled oakmoss with reduced allergenic potential can be prepared. Consequently a highly selective and specific analytical method is necessary to support this effort. This is not available with the existing HPLC methods using UV detection.

Keywords: Oakmoss; Atranorin; Allergens

1. Introduction

Oakmoss absolute is derived from the lichen *Evernia prunastri* and grows on oak trees primarily in central Europe. The process for preparing oakmoss absolute traditionally involves extracting the harvested oakmoss with hydrocarbon solvents that include benzene. The residue from this extract is subsequently extracted with an alcohol to produce the desired product [1,2]. Benzene is no longer used and has been replaced by more polar hydrocarbons. This has resulted in a change in the composition of oakmoss.

Oakmoss absolute is ubiquitous in the fragrance industry and is found in after-shave lotions, cosmetics and fine perfumes. It is valued for the woody aroma it imparts and for its fixative properties. However, since 1948 oakmoss has been known to cause an allergic response in some individuals [3]. Since this initial investigation, other researchers have verified this finding and have attempted to isolate the cause of the allergic response to specific compounds [4–7]. The phenylbenzoates, atranorin, chloroatanorin and evernic acid, were found to cause an allergic response in some individuals when benzene was used in the oakmoss process. Additionally, fumarprotocetraric acid, cetraric acid and stictic acid, which have a phenylbenzoate functionality config-

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ured in a cyclic system were also implicated. Although not a phenylbenzoate, usnic acid was also determined to cause an allergic response [8–11].

In addition to a phenylbenzoate functionality, atranorin, chloroatranorin, fumarprotocetraric acid and cetraric acid also contain an aldehyde functionality. Both aldehydes and phenylbenzoates are known to cause an allergic response by forming covalent complexes with skin proteins [12–16]. Also, esters formed by transesterification during the thermal decomposition of atranorin and chloroatranorin during the oakmoss process have been shown to cause an allergic response. Terajima et al. [17] found that ethyl chlorohematommate, which is formed by transesterification of chloroatranorin from the phenyl ester to the ethyl ester, is an allergen present in oakmoss absolute.

Current research is focused toward the development of a reduced allergenic oakmoss absolute [18–20]. This involves the use of preparative high-performance liquid chromatography (HPLC), catalytic hydrogenation, alkaline treatment and formation of Schiff bases to remove atranorin and chloroatranorin and also ethyl hematommate, ethyl chlorohematommate, atranol and chloroatranol which are byproducts formed by transesterification or decarboxylation of atranorin and chloroatranorin during the oakmoss process. The challenge is to do this without affecting the aroma quality of oakmoss absolute. A number of HPLC methods have been developed for the analysis of components in oakmoss and other lichens [21–25]. These are predominately reversed-phase methods using an octadecylsilane (C_{18}) HPLC column with a mobile phase consisting of water acidified with phosphoric or acetic acid with methanol or acetonitrile as the organic modifier. Gradient elution is used with UV detection either at a fixed wavelength or using a photodiode array detector. Oakmoss absolute is a complex mixture of components all of which cannot be easily separated by a single HPLC method using UV detection. Feige et al. [26] clearly demonstrated that many lichen compounds coelute. HPLC interfaced with tandem mass spectrometry (LC–MS–MS) introduces a second dimension of separation, resulting in greater specificity and selectivity, necessary for the accurate identification of components in such a complex mixture.

This paper describes an LC–MS–MS method for the accurate identification of atranorin and related components with potential allergenicity. This method enables a high degree of selectivity and specificity that is necessary to support the development of a reduced allergenic oakmoss absolute and that is not available with previously reported HPLC methods using UV detection. A review of the literature shows this to be the first reported LC–MS–MS method for the identification of atranorin and related potential allergens in oakmoss absolute. Future work is focused toward expanding this qualitative method to a quantitative method. If greater sensitivity is required, the work described in this paper has provided the groundwork for developing a quantitative LC–MS–MS method utilizing selected reaction monitoring (SRM).

2. Experimental

2.1. Chemicals

Atranorin, which is an approximately 50:50 mixture of atranorin and chloroatranorin, was purchased from Aldrich (Milwaukee, WI, USA). L-Methionyl-arginyl-phenylalanyl-alanine acetate (MRFA) and apomyoglobin were purchased from Sigma (St. Louis, MO, USA). All other solvents were HPLC grade and were purchased from Fisher Scientific (Springfield, NJ, USA). Atranol, chloroatranol, ethyl hematommate and ethyl chlorohematommate were synthesized by Dr. Daniel Joulain (Robertet, Grasse, France).

2.2. Sample preparation

Oakmoss absolute solutions containing 10 mg/ml were prepared in methanol. A solution containing 250 μg/ml each of atranol, chloroatranol, ethyl hematommate and ethyl chlorohematommate was prepared in methanol. A solution of atranorin–chloroatranorin containing 600 μg/ml was prepared in acetonitrile–dimethylformamide (80:20). The atranorin–chloroatranorin solution had to be sonicated to dissolve. All solutions were filtered prior to analysis.

A solution containing 5 pmol/μl apomyoglobin plus 20 pmol/μl of MRFA was prepared in methanol–water (50:50) containing 0.5% acetic acid.

2.3. Liquid chromatography

A SpectraSystem P4000 gradient pump and AS3000 autosampler (Thermo Separation Products, San Jose, CA, USA) were used for this work. Mobile phase A consisted of water with 2% acetic acid and mobile phase B consisted of acetonitrile with 2% acetic acid. A gradient was run from 10% B to 95% B in 30 min at a flow-rate of 0.2 ml/min. The mobile phase composition was held at 95% B for 10 min for a total run time of 40 min. The column was equilibrated at initial mobile phase conditions for 10 min at the end of each analysis. A 150 mm×2.1 mm Zorbax, 5 μm, SB-C_{18} narrow bore HPLC column (MAC-MOD Analytical, Chadds Ford, PA, USA) was used with a guard column (Upchurch Scientific, Oak Harbor, WA, USA). The column was operated at ambient temperature and the injection volume was 10 μl.

2.4. Mass spectrometry

A Finnigan TSQ 7000 (Finnigan, San Jose, CA, USA) triple quadrupole mass spectrometer with an API2 source was interfaced to the liquid chromatograph. The mass spectrometer was operated in the negative ion mode using atmospheric pressure chemical ionization (APCI). The vaporizer and heated capillary temperatures were 300°C and 250°C, respectively. The corona discharge was set to 5 μA (3.5 kV). The sheath gas was set to 60 p.s.i. (1 p.s.i.=6894.76 Pa). The auxiliary gas was not used. Full scan LC–MS spectra were obtained by scanning Q1 from 140 to 600 amu in 1 s. The electron multiplier was optimized for a gain of 300 000 (1030 V).

MS–MS spectra for oakmoss absolute samples were obtained by using Q1 to sequentially focus selected ions into the collision cell (Q2) which was maintained at 2 mT with argon. The offset voltage for Q2 was varied to optimize the spectra for the individual compounds. The Xcalibur data system was set up with the following segments to accomplish this:

Segment	Segment time (min)	Set mass (u)	Q2 offset voltage (V)	Analyte
1	16.0	151.0	25	Atranol
2	2.5	185.1	25	Chloroatranol
3	3.4	208.9	25	Methyl hematommate
4	1.4	242.8	25	Methyl chlorohematommate
5	1.4	223.0	25	Ethyl hematommate
6	1.2	257.0	25	Ethyl chlorohematommate
7	1.0	237.0	25	Isopropyl hematommate
8	1.9	270.9	25	Isopropyl chlorohematommate
9	1.3	373.1	17	Atranorin
10	9.9	407.0	17	Chloroatranorin

The peakwidth for Q1 was increased to 4 amu and the electron multiplier increased to 1330 V to enhance sensitivity. Q3 was scanned from 20 to 450 amu in 1 s.

MS–MS spectra for atranorin, chloroatranorin, ethyl hematommate, ethyl chlorohematommate, atranol and chloroatranol were obtained by infusing a solution containing standards for each component into the mobile phase stream at 10 μl/min, using a Harvard Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). The mobile phase

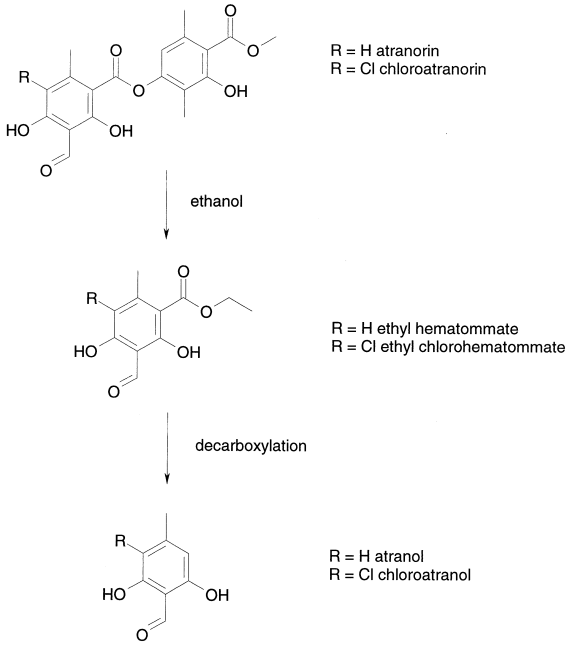

Fig. 1. Thermal decomposition products of atranorin and chloroatranorin formed during the oakmoss process.

flow-rate was 0.2 ml/min and the ratio of mobile phase A to mobile phase B was 50:50.

Q1 and Q3 were tuned and calibrated with MRFA–apomyoglobin. Subsequent to the initial tuning and calibration, the vaporizer and capillary temperatures and sheath and auxiliary gas pressures were set to maximize the total ion current (TIC) at a mass-to-charge ratio (m/z) of 373 while infusing a solution of atranorin into the mobile phase stream at 10 μl/min. The mobile phase flow-rate was 0.2 ml/min and the ratio of mobile phase A to mobile phase B was 50:50. Additionally, the capillary

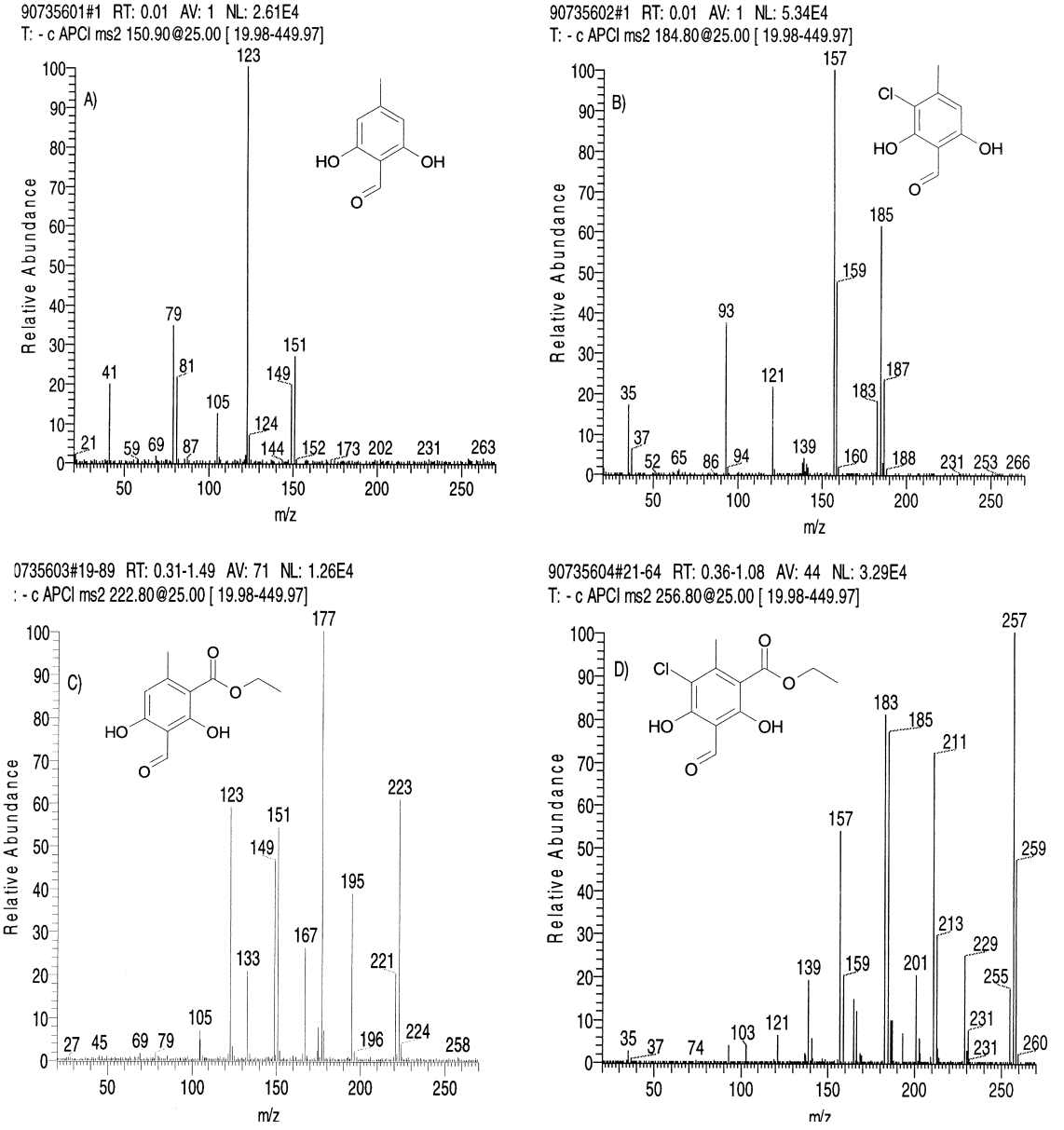

Fig. 2. Negative ion MS–MS spectra of (A) atranol (M_r 152), (B) chloroatranol (M_r 186), (C) ethyl hematommate (M_r 224), and (D) ethyl chlorohematommate (M_r 258).

voltage and lens voltages were optimized for atranorin.

3. Results and discussion

Ethyl hematommate, ethyl chlorohematommate, atranol and chloroatranol are formed by transesterification and decarboxylation of atranorin and chloroatranorin during the oakmoss process (Fig. 1). This occurs largely because of the thermal lability of atranorin and chloroatranorin [27].

The full scan LC–MS negative ion mass spectra of chloroatranorin, atranorin, ethyl chlorohematommate, ethyl hematommate, chloroatranol and atranol display the deprotonated molecules $[M-H]^-$ detected at m/z 407, 373, 257, 223, 185 and 151, respectively. Minor adduct ions $[M+CH_3COO]^-$ were also detected. Minor fragment ions were detected at m/z 151 and 195 for atranorin and correspond to the atranol moiety and cleavage of the ester to form the carboxylate anion, respectively. The corresponding fragment ions for chloroatranorin were detected at m/z 185 and 229. These fragmentations are more prevalent at higher vaporizer temperatures and are likely due to the thermal lability of these compounds. At a vaporizer temperature of 450°C the base peak in the LC–MS mass spectrum of atranorin is observed at m/z 195 while that for chloroatranorin is observed at m/z 185 along with numerous other high-intensity fragment ions. Since this results in a decrease in the total ion current for $[M-H]^-$ there is less sensitivity for acquiring MS–MS spectra at higher vaporizer temperatures. This is the reason a vaporizer temperature of 300°C was chosen for this analysis. Furthermore, the TIC was monitored at m/z 375 in the positive ion mode and at m/z 373 in the negative ion mode to determine that the response for atranorin was greatest in the negative ion mode.

The MS–MS spectra for atranol, chloroatranol, ethyl hematommate and ethyl chlorohematommate (Fig. 2) result largely from the loss of small neutral molecules from $[M-H]^-$. For instance, the $[M-H]^-$ for ethyl chlorohematommate loses CO and ethanol to form the fragment ions at m/z 229 and 211, respectively (Fig. 2D). Sequential loss of HCl and CO from the ion at m/z 229 yields the ions observed at m/z 193 and 165, respectively. Additionally, $[M-H]^-$ for ethyl chlorohematommate loses ethylene to form the carboxylate anion at m/z 229 followed by loss of CO_2 to form the fragment ion at m/z 185. The fragment ion at m/z 185 is likely to be structurally similar to the $[M-H]^-$ for chloroatranol (Fig. 2B). It undergoes sequential loss of CO and H_2O to form the ions at m/z 157 and 139. Evidence for this sequence is manifested by the presence of ^{37}Cl species at m/z 159 and 141. The fragment ion at m/z 157 loses HCl to form the ion at m/z 121.

The fragment ion at m/z 177, in the MS–MS spectrum of atranorin, is due to cleavage adjacent to the carbonyl group forming a neutral phenol (Fig. 3A). The comparable fragmentation in chloroatranol (Fig. 3B) produces the fragment ion at m/z 211. Fragmentation by a similar mechanism yields the phenoxide anion at m/z 195 in both compounds.

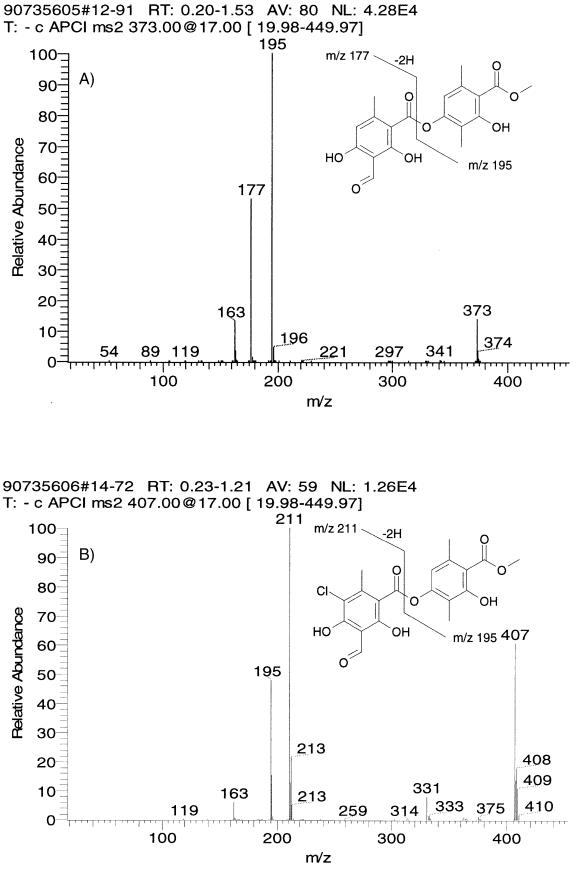

Fig. 3. Negative ion MS–MS spectra of (A) atranorin (M_r 374) and (B) chloroatranorin (M_r 408).

Loss of methanol from m/z 195 yields the ion at m/z 163. At higher collision energies, loss of CO from m/z 211 and 163 in chloroatranorin and from m/z 177 and 163 in atranorin would likely be observed. The ion at m/z 331 in the MS–MS spectrum of chloroatranol is due to sequential loss of methanol and CO_2 from $[M-H]^-$. This is also observed in the MS–MS spectrum of atranorin but to a lesser extent.

Based on the fragmentation patterns for the standard compounds, four additional related compounds were identified. These are methyl and isopropyl hematommate and methyl and isopropyl chlorohematommate. These were first identified in our laboratory by gas chromatography–mass spectrometry (GC–MS) [28]. Additionally, a second atranol isomer was identified. This suggests the possibility of a second atranorin isomer but it was not detected in the samples analyzed. Fig. 4 contains a TIC for oakmoss absolute in the LC–MS mode and a TIC acquired in LC–MS–MS mode showing the identified compounds.

Although there were similar fragmentations in the MS–MS spectra for the two homologous series, there were also some interesting differences. The formation of the fragment ion corresponding to the chloroatranol moiety at m/z 185 was greater for isopropyl chlorohematommate than for ethyl chlorohematommate and was not detected in methyl chlorohematommate (Fig. 5). This is because the tendency of isopropyl chlorohematommate to lose propene, to form the carboxylate anion precursor (m/z 229) to m/z 185, is greater than the tendency for ethyl chlorohematommate to lose ethylene in forming the same precursor. This mechanism is not possible for methyl chlorohematommate and the base peak at m/z 183 is formed through sequential loss of

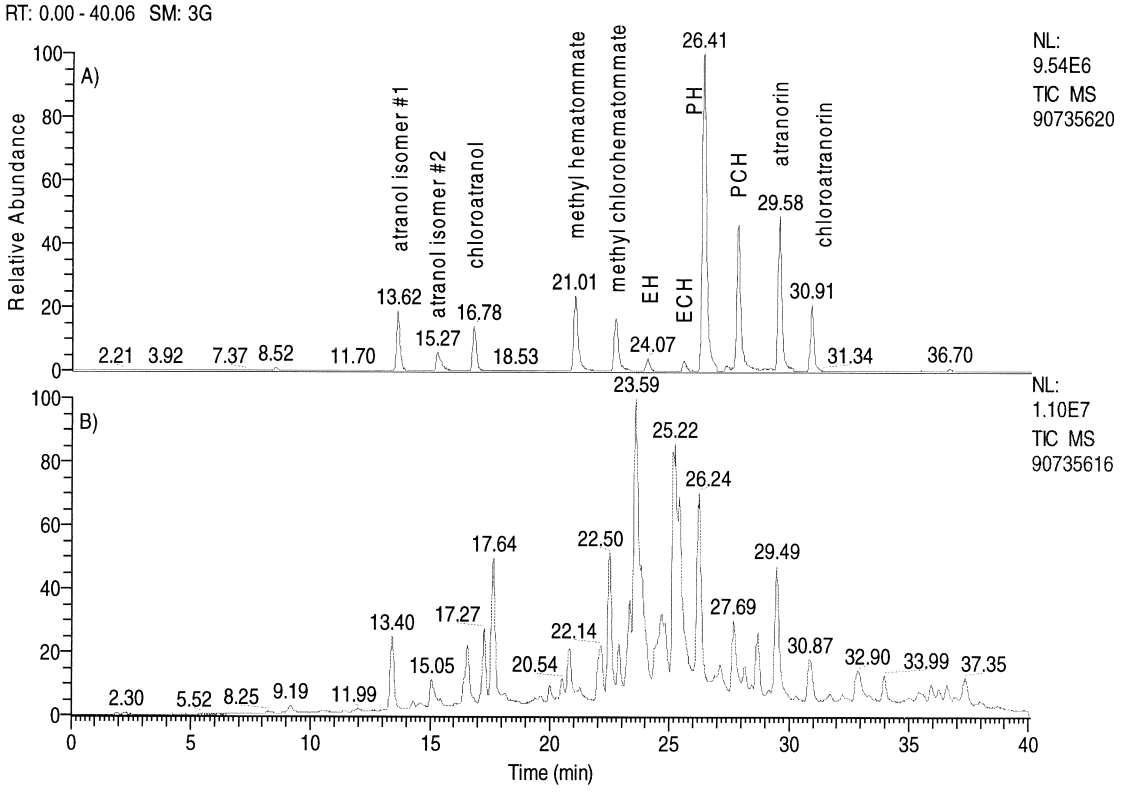

Fig. 4. (A) LC–MS–MS TIC of atranol isomers, chloroatranol, methyl hematommate, methyl chlorohematommate, ethyl hematommate (EH), ethyl chlorohematommate (ECH), isopropyl hematommate (PH), isopropyl chlorohematommate (PCH), atranorin and chloroatranorin. (B) LC–MS TIC of oakmoss absolute.

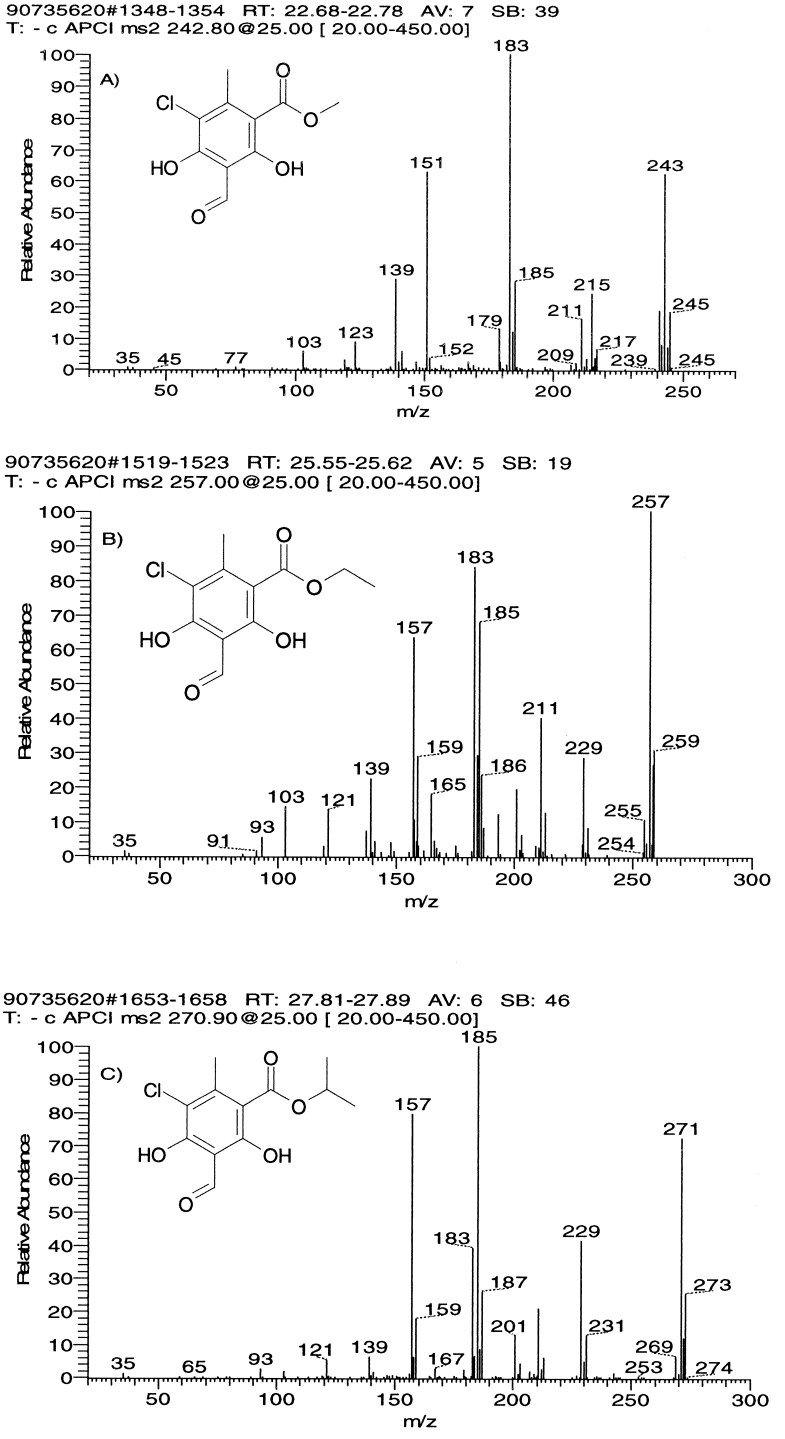

Fig. 5. Negative ion LC–MS–MS spectra of the methyl (M_r 244), ethyl (M_r 258), and isopropyl chlorohematommate (M_r 272) homologous series.

methanol and CO from $[M-H]^-$. Loss of the corresponding alcohol, from the ester moiety, followed by loss of CO is evident in all three homologues but may be more pronounced in methyl chlorohematommate because the mechanism for formation of the carboxylate anion at m/z 229 is not possible. A major fragmentation route for methyl chlorohematommate is sequential loss of CO, HCl and CO to form the fragment ions at m/z 215, 179 and 151, respectively. This sequence is observed in the MS–MS spectrum of ethyl chlorohematommate leading to ions at m/z 229, 193 and 165 and is a minor fragmentation route for isopropyl chlorohematommate. Comparable fragmentation mechanisms are seen for the homologous series methyl, ethyl and isopropyl hematommate.

Evernic acid was not detected in the samples analyzed. However, the decomposition products 2,4-dihydroxy-6-methyl ethylbenzoate and 2-hydroxy-4-methoxy-6-methyl methylbenzoate were tentatively identified. Evernic acid is also known to be thermally labile and likely decomposes during the oakmoss process [21]. Stictic, cetraric and fumarprotocetraric acids were also not identified in any of the samples analyzed.

Usnic acid was tentatively identified. Its MS–MS spectrum is shown in Fig. 6. This spectrum is unusual because the ion at m/z 328 suggests the formation of an odd-electron ion $[M-CH_3-H]^-$ from an even-electron ion $[M-H]^-$ in a low-energy collision cell. This violates the even-electron rule [29]. However, Karlsson et al. reported the observation of an odd-electron ion $[M-CH_3+H]^+$ in their work with sphingolipids [30] and Chen et al. reported the formation of odd-electron ions from even-electron precursors in their work with catecholamine derivatives [31].

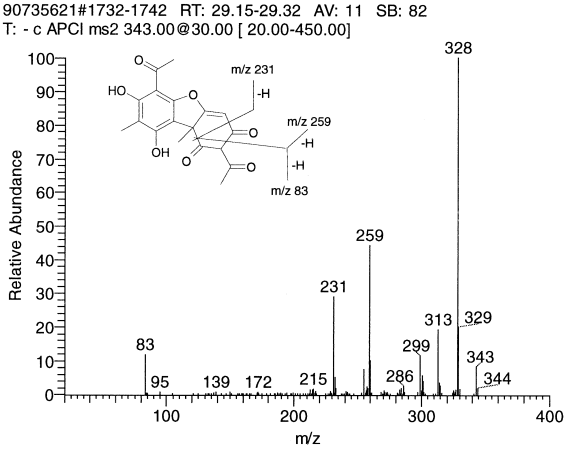

Fig. 6. Negative ion LC–MS–MS spectrum of compound tentatively identified as usnic acid (M_r 344).

4. Conclusions

A highly selective and specific method was developed that enables the accurate identification of atranorin and related potential allergens in oakmoss absolute. This is a significant improvement over existing HPLC methods using UV detection, either at a single wavelength or with a photodiode array detector, because there is a second dimension of separation implicit in an LC–MS–MS experiment. Also, MS–MS spectra are rich in spectral detail enabling structural elucidation. This facilitates the accurate identification of the compounds causing an allergic response so a quality controlled oakmoss with reduced allergenic potential can be prepared. A second atranol isomer was also identified which suggests the possibility of a second atranorin isomer. This may be of interest in the taxonomic investigation of lichens. A review of the literature shows this to be the first LC–MS–MS method for the identification of atranorin and related potential allergens in oakmoss absolute. Future work is focused toward expanding this qualitative method to a quantitative method. This will enable the establishment of a standard for reduced allergenic oakmoss.

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