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Analysis of fresh triterpenoid resins and aged triterpenoid varnishes by high-performance liquid chromatography–atmospheric pressure chemical ionisation (tandem) mass spectrometry

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Abstract

Fresh triterpenoid (dammar and mastic) resins and aged triterpenoid varnishes were analysed by on-line HPLC–MS using atmospheric pressure chemical ionisation (APCI). Twenty components were identified in the fresh resins [dammarenolic acid, ursonic acid, oleanonic acid, hydroxydammarenone (I and II), dammaradienol, oleanonic aldehyde, dammarenediol, ursonic aldehyde, oleanolic aldehyde, ursolic aldehyde, hydroxyhopanone, dammaradienone, moronic acid, (3L,8*R*)-3,8-dihydroxypolypoda-13*E*,17*E*,21-triene, (8*R*)-3-oxo-8-hydroxypolypoda-13*E*,17*E*,21-triene, masticadienonic acid, isomasticadienonic acid, 3-*O*-acetyl-3-epimasticadienolic acid and 3-*O*-acetyl-3-epiisomasticadienolic acid]. Analysis of the aged varnishes revealed the presence of some oxidised triterpenoid components [11-oxo-oleanonic acid, 11-oxo-oleanonic acid, 3-oxo-25,26,27-trinordammarano-24,20-lactone, 20,24-epoxy-25-hydroxy-3,4-seco-4(28)dammaren-3-oic acid and some ocotillone type molecules]. Most constituents of these complex samples were well resolved by reversed-phase HPLC. APCI-MS provides useful information about the molecular mass and the presence of certain functional groups. Specific marker compounds were found, which enable the discrimination between aged dammar and mastic varnishes by HPLC–APCI-MS. The fragmentation behaviour of triterpenoids under APCI conditions was compared to that under electron impact conditions. Mass spectrometric fragmentation of triterpenoids with a saturated ring system is straightforward. Triterpenoids with an unsaturation in the ring system probably give rise to double bond migration. Subsequent low energy MS–MS analysis does not provide more structural information, since mainly non-specific ring fragment ions are formed.

Keywords: Varnishes; Dammar; Mastic; Art analysis; Triterpenoids; Terpenoids

1. Introduction

Terpenoids, which are widely distributed in nature in both the plant and animal kingdom, are made up of units of the C_5 compound isoprene. Triterpenoids are C_{30} alicyclic compounds which often contain several functional groups. Triterpenoid resins are used as varnishes on paintings, in order to increase the colour saturation and gloss, and to protect against ageing of the paint surface. However, triterpenoid

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varnishes deteriorate in the course of time, leading to yellowed and brittle products. Our research focuses on the molecular ageing process of two resins which are frequently used as picture varnishes, the triterpenoid resins dammar (originating from the trees of the Dipterocarpaceae) and mastic [the bleed resin of *Pistacia lentiscus* L. (Anacardiaceae)]. Both resins mainly consist of triterpenoids together with a smaller proportion of polymeric material. The molecular composition of fresh dammar and mastic resin has been investigated by several research groups [1–20].

In previous work, it was shown that gas chromatography–mass spectrometry (GC–MS) after methylation does not resolve the constituents of aged varnishes [21]. Since it is likely that both oxidation and condensation reactions take place during the ageing process, GC–MS is not the most appropriate method for the elucidation of all ageing products. High-performance liquid chromatography (HPLC) is suitable for the analysis of a much broader range of compounds. In addition, thermolabile compounds can be analysed and no chemical derivatisation is required.

In earlier work [21], HPLC fractions were identified by subsequent analysis with GC–MS. Since off-line HPLC–GC–MS is very time-consuming and volatility of the constituents is still required, on-line HPLC–MS is an interesting alternative. Furthermore, additional MS–MS data can be used for the purpose of molecular identification. Moderately polar samples that are not too labile can be analysed by on-line HPLC using atmospheric pressure chemical ionization (APCI) [22]. APCI-MS(–MS) is often used as a very sensitive detector, especially in the area of biomedical research by applying selected-ion monitoring (SIM) and multiple-reaction monitoring (MRM) [23–26]. Relatively little attention has been paid to the fundamental aspects of fragmentation under APCI conditions. Sample ions may fragment, once they have left the ion source and have been extracted into the vacuum system. As the gas pressure is still relatively high at this point and the ions are being accelerated, suitable conditions are created for collision induced dissociation (CID). This CID ''up front'' is very easy and reproducible and in some cases it has been used for the purpose of structural elucidation [22,27–29].

In this study, comparative HPLC–MS and HPLC–MS–MS data are presented on fresh resins and aged dammar and mastic varnishes. The fragmentation behaviour of triterpenoids under APCI conditions is studied by comparing mass spectra obtained with APCI-MS and APCI–MS–MS with data on the same compounds that were already identified with GC–MS [electron impact (EI) ionisation], because fragmentation patterns are better understood under EI conditions [30–34].

2. Experimental

2.1. Materials

Oleanolic acid was purchased from Aldrich. Methanolic extracts (5 mg/ml) were prepared of Dammar (A.J. van der Linde, Amsterdam, The Netherlands) and Mastic (H. Schmincke, Erkrath, Germany). The aged dammar varnish was removed from the painting ''Stadsgezicht'' by Metzelaar (private ownership) by using a swab with propan-2-ol (isopropanol). The varnish was analysed after redissolution in isopropanol. The aged mastic varnish was removed from the painting ''Supper at Emmaus'' by Jan Steen (Rijksmuseum, inv. No. SK-A-1932) by applying a mixture of acetone–mineral spirits (70:30) to the varnish layer. After pressing down a sheet of polyester film on the surface, the varnish was removed mechanically (''peeled off''). The varnish was scraped off from the polyester film and dissolved in methanol for analysis. It was not known when these varnishes were applied on the paintings. Both the fresh resins and the aged varnishes were subjected to HPLC–APCI-MS(–MS) and HPLC in combination with GC–MS (Fig. 1).

2.2. HPLC

The HPLC equipment consisted of a solvent-delivery system (HP1090, Hewlett-Packard) and a Rheodyne 7125 injection valve equipped with a 20-μl loop, connected to a C_{18} column (Merck: LiChrospher 100 RP-18, 5 μm, 250×4 mm I.D.), which was kept at 35°C. For the analysis of the fresh resins, eluent A consisted of a mixture of water–acetonitrile (20:80), eluent B was a mixture of water–acetonitrile (2:98) and eluent C was acetonitrile. Separation was

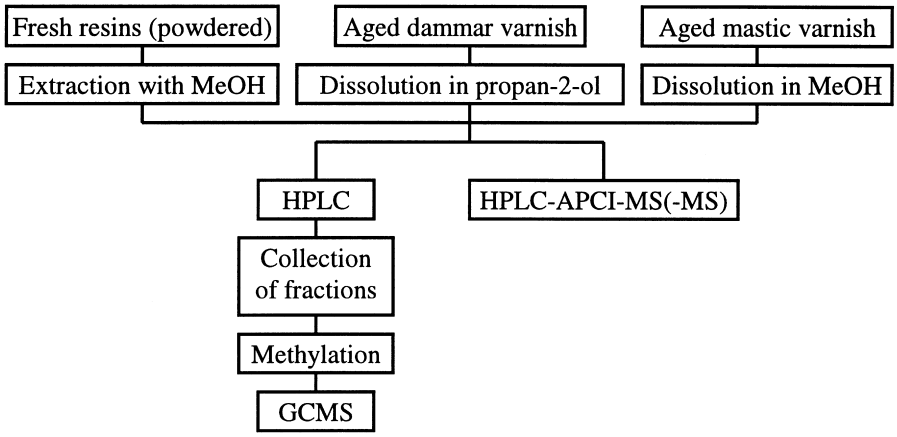


Fig. 1. Analysis scheme for the fresh resins and aged varnishes.

achieved with a linear gradient from A to B in 30 min, followed by an isocratic period of 9 min, going to eluent C in 1 min, followed by an second isocratic period of 10 min using a flow-rate of 0.8 ml/min. For the analysis of the aged varnishes, eluent A was a mixture of water–acetonitrile (80:20), eluent B was acetonitrile. Separation was achieved with a linear gradient from A to B in 60 min, followed by an isocratic period of 15 min, using a flow-rate of 0.8 ml/min. The HPLC fractions of the fresh resins and the aged varnishes were collected and subsequently analysed by GC–MS after methylation.

2.3. APCI-MS(–MS)

The outlet from the HPLC system was connected directly to the APCI interface of either a VG Quattro (Figs. 7,9,11) or a VG Quattro II (Figs. 2,4–6) mass spectrometer (Micromass/Fisons Instruments). For system control and data processing, MassLynx software (Micromass/Fisons VG) was used. The source and APCI probe temperatures were maintained at 150°C and 350°C, respectively, and the corona discharge was kept at 3.5 kV. The cone voltage was set at 20 V (in the case of Figs. 2,4–6d, Fig. 9b) or at 30 V (in the case of Figs. 7 and 11). HPLC–MS–MS experiments were carried out using argon at a pressure of $1.7 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$ mbar and a collision energy of 40 V (in case of Figs. 7 and 11) or 50 V (in case of Fig. 9).

2.4. GC–MS

For methylation, according to the method of Hashimoto et al. [35], the fractions were first evaporated to dryness. A 250-μl volume of methanol, 25 μl of benzene and 10 μl of trimethylsilyl (TMS) diazomethane were added. This mixture was left at room temperature for 30 min. After evaporation to

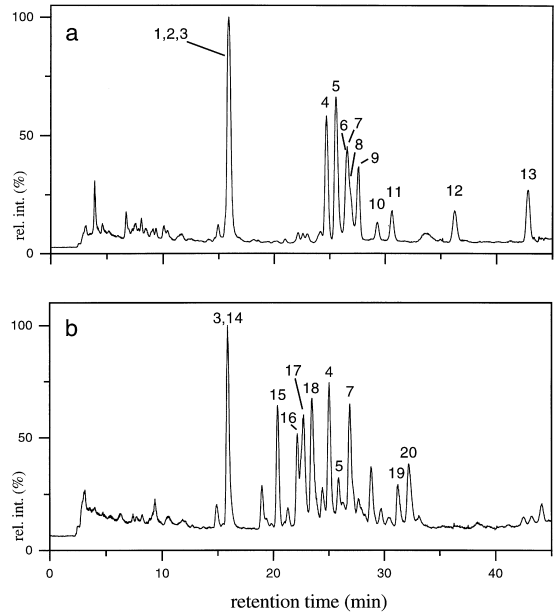


Fig. 2. Total ion current traces of reversed-phase HPLC–APCI-MS, using the positive ion mode, of fresh dammar (a) and mastic (b) resin (peak labels refer to Table 1).

dryness, the sample was dissolved in dichloromethane. GC–MS data were obtained with a fused-silica BPX5 column (SGE) (25 m×0.32 mm I.D., 0.25 μm film thickness) in a gas chromatograph (Carlo Erba, series 8565 HRGC Mega 2) coupled directly to the ion source of a JEOL DX-303 double focusing mass spectrometer (E/B). Helium was used as the carrier gas with a linear velocity of approximately 26 cm/s. The temperature was programmed for 2 min at 50°C, subsequently to 250°C at a rate of 8°C and from 250°C to 350°C at a rate of 3°C/min. A Jeol MP-7000 data system was used for data acquisition and processing. The mass spectrometer was scanned from m/z 40–700 with a 1 s cycle time. Ions were generated by EI (70 eV), extracted at 3 kV and postaccelerated to 10 kV. The MS information was interpreted and compared with spectra available in the literature.

3. Results and discussion

3.1. Separation of constituents of fresh triterpenoid resins by reversed-phase HPLC

In order to remove the polymeric fractions which are present in both fresh dammar and mastic resin, methanolic extracts were analysed in which only the triterpenoid part of the resins dissolved. Fig. 2 depicts the reversed-phase HPLC–APCI-MS total ion currents (TICs) of fresh dammar (a) and mastic (b) resin, using the positive ion mode and an acetonitrile–water eluent system. In order to identify the constituents of the resins by their EI (70 eV) spectra, [9,11,17,19,30–34], HPLC fractions were collected and subsequently analysed by GC–MS (off-line HPLC–GC–MS). Table 1 lists the compounds identified, their molecular mass (M_r) and their mass spectrometric characteristics under both EI (corresponding methylated compounds) and APCI conditions. All (fragment) ion peaks that were found under APCI conditions, with an intensity higher than 10% of the base peak, are listed. In some cases, fragment ion peaks with an intensity lower than 10% of the base peak which are useful for identification purposes are also shown. In case of compounds with the oleanane or ursane skeleton, not all fragment ion peaks around m/z 200 are given in Table 1, since these peaks were observed not to reproduce well. This observation will be described in Section 3.2. The molecular structures of the identified compounds are shown in Fig. 3. Two compounds, which have not been found before in dammar resin, i.e., oleanolic and ursolic aldehyde (10 and 11), were identified by their EI spectrum. It is evident that some of the triterpenoids which contain an acid group (compounds 1, 2, 3 and 14) in fresh dammar as well as in mastic resin are not well resolved by this acetonitrile–water eluent system. The addition of a buffer may improve the separation. The other triterpenoids with hydroxyl, keto and/or aldehyde groups are well separated. Interestingly, the two stereoisomers hydroxydammarenone I and II (4 and 5), which are abundantly present in dammar resin, are well separated by reversed-phase HPLC. This separation could not be achieved by GC–MS [21].

3.2. Separation of constituents of aged triterpenoid varnishes by reversed-phase HPLC

Complex mixtures, which contain polar species, are likely to be formed by oxidation reactions during the ageing process of triterpenoid varnishes [21]. The reversed-phase HPLC–APCI-MS TICs of an aged dammar varnish and an aged mastic varnish (which also contains some components of another type of varnish made from diterpenoid resin) are shown in Fig. 4a (dammar) and Fig. 4b (mastic). The peak labels refer to those used in Table 1 and Fig. 3. Since more polar compounds are expected to be formed by ageing, a more polar eluent is used to start the HPLC gradient in the analysis of the aged varnishes, compared to the eluents used to analyse the fresh resins. The constituents of the aged varnishes are well resolved by reversed-phase HPLC, compared to the separation achieved by GC–MS [21]. The concentrations of the samples of the fresh resin extracts and the aged varnishes were in the same order of magnitude (in the order of a few micrograms per millilitre). Determination of the exact concentration of triterpenoid varnish solutions is difficult for a number of reasons. For example, it is not known what kind of varnish preparation was used originally, whether certain additives were deposited on top of the varnish layer by painting restorers, or whether certain varnish constituents volatilize during ageing.

Table 1
List of compounds identified in fresh triterpenoid resins and aged varnishes

Label	Compound name	M_r	*m/z* Values of characetristic (fragment) ions of methylated compound under EI (70 eV) (rel. int., %)	*m/z* values of characteristic (fragment) ions of compounds under APCI (cone 20 V) (rel. int., %)
1	Dammarenolic acid (20[a]-hydroxy-3,4-seco-4(28),24-dammaradien-3-oic acid)	458	454(50), 385(48), 109(100)	441(100), 205(23), 191(44)
2	Ursonic acid (3-oxo-12-ursen-28-oic acid)	454	468(17), 262(100), 203(76), 133(41)	455(100), 437, 409[b]
3	Oleanonic acid (3-oxo-olean-12-en-28-oic acid)	454	468(25), 262(53), 203(100)	455, 437, 409(100)[b]
4	Hydroxydammarenone (I or II) (20[a]-hydroxy-24-dammaren-3-one)	442	424(100), 355(44), 205(60), 109(91)	443(6), 425(100), 407(9), 219(21), 205(13)
5	Hydroxydammarenone (I or II) (20[a]-hydroxy-24-dammaren-3-one)	442	424(100), 355(45), 205(73), 109(99)	443(6), 425(100), 407(11), 219(21), 205(12)
6	Dammarendiol (20[a]-dammar-24-ene-3β,20-diol)	444	425(80), 408(17), 207(53), 189(60), 109(100)	427(30), 409(100), 219(20), 191(34)
7	Oleanonic aldehyde (3-oxo-olean-12-en-28-al)	438	438(26), 232(52), 203(100)	439(100), 421(27), 411(9), 393(3)
8	Dammaradienol (3β-hydroxy-20,24-dammarediene)	426	426(84), 408(16), 207 (76), 189(54), 109(100)	427(32), 409(100), 219(22), 191(42)
9	Ursonic aldehyde (3-oxo-urs-12-en-28-al)	438	438(16), 232(21), 203(100)	439(100), 421(24), 411(17), 393(3)
10	Oleanolic aldehyde (3-hydroxy-olean-12-en-28-al)	440	440(9), 232(77), 203(100)	441(29), 423(100), 395(25), 205(14), 191(29)
11	Ursolic aldehyde (3-hydroxy-urs-12-en-28-al)	440	440(7), 232(27), 203(100)	441(41), 423(100), 395(26), 205(16), 191(34)
12	Hydroxyhopanone 21β,22-hydroxy-3-hopanone)	442	442(20), 424(18), 409(14), 384(38), 207(58), 189(87), 149(100)	443(18), 425(100), 407(12), 179(10)
13	Dammaradienone [3-oxo-dammara-20(21),24-diene]	424	424(100), 205(66), 109(82)	425(100), 407(34), 245(24), 189(19)
14	Moronic acid (3-oxo-olean-18-en-oic acid)	454	468(61), 249(55), 189(100)	455(100), 437, 409[b]
15	(3L,8*R*)-3,8,-Dihydroxypolypoda-13*E*,17*E*,21-triene	444	426(7), 218(31), 208(33), 190(45), 175(43), 137(59), 94(62), 81(97), 69(100)	427(58), 409(100)
16	(8*R*)-3-Oxo-8-hydroxypolypoda-13*E*,17*E*,21-triene	442	424(9), 218(29), 175(24), 137(52), 94(53), 81(100), 69(90)	425(100), 407(7), 219(10), 205(18), 191(13)
17	(Iso)masticadienonic acid (3-oxo-13α,14β,17βH,20αH-lanosta-8,24-dien-26-oic acid or 3-oxo-13α,14β,16βH,20αH-lanosta-7,24-dien-26-oic acid)	454	468(31), 453(100), 421(21)455(100), 437(45)	
18	idem	454	468(28), 453(100), 421(19)	455(98), 437(100), 127(20), 125(27)

(Cont.)

Table 1 (continued)

Label	Compound name	M_r	m/z Values of characetristic (fragment) ions of methylated compound under EI (70 eV) (rel. int., %)	m/z values of characteristic (fragment) ions of compounds under APCI (cone 20 V) (rel. int., %)
19	3-*O*-Acetyl-3epi(iso)masticadienolic acid[c] (3α-acetoxy-13α,14β,17βH,20αH-lanosta-8,24-dien-26-oic acid or 3α-acetoxy-13α,14β,17βH,20αH-lanosta-7,24-dien-26-oic acid)	498	512(24), 497(40), 437(100)	439(100), 247(17), 191(56)
20	idem	498	512(26), 497(29), 437(100)	439(100)
21	11-Oxooleanonic acid (3,11-dioxo-olean-12-en-28-oic acid)	468	482(100), 317(51), 276(97), 257(50), 217(69)	469(100), 451(4), 423(29)
22	11-Oxo-ursonic acid (3,11-dioxo-urs-12-en-28-oic acid)	468	482(90), 317(100), 276(42), 257(31)	469(100), 451, 423[b]
23	Oxidised oleanane type molecule	?	468(90), 263(69), 233(100)	469(100), 451(61), 423(49), 263(8), 233(37)
24	3-Oxo-25,26,27-trinordammarano-24,20[a]-lactone	414	414(100), 315(30), 205(51), 99(45), 95(33)	415(100), 397(31), 379(4)
25	Oxidised ursane type molecule	?	468(58), 263(80), 233(100)	469(100), 451(11), 423(28), 263(2), 233(27)
26	20,24-Epoxy-25-hydroxy-3,4-seco-4(28)dammaren-3-oic acid[d]	474	429(10), 143(100)	457[b], 439(100)

Peak labels correspond to those used in Figs. 2–4. The molecular mass (M_r), the characteristic m/z values of the compounds under APCI-MS conditions and the characteristic m/z values under EI (70 eV) conditions (corresponding methylated compounds) are listed. All (fragment) ion peaks that were found under APCI conditions, with an intensity higher than 10% of the base peak, are listed. In some cases, fragment ion peaks with an intensity lower than 10% of the base peak which are useful for identification purposes are also shown.

[a] The configuration at C20 was not determined.

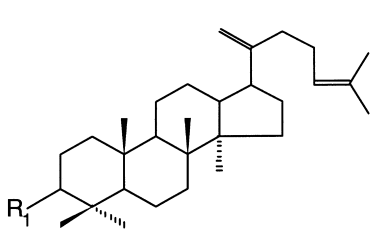
[b] The exact relative intensities could not be determined since this compound could not be separated from other compounds with the HPLC conditions used here.

[c] The identifications were confirmed by LC–APCI-MS in the negative ion mode, which produced spectra with only one ion, m/z 498.

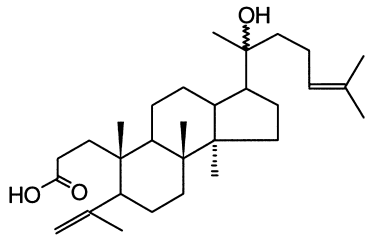
[d] The configuration at C20 and C24 was not determined. Two stereoisomers were separated by HPLC under the chosen conditions.

When comparing the signal-to-noise ratio of the HPLC–MS results of the aged varnishes to that of the fresh resins (Fig. 2), it is clear that only a small part of the aged varnishes is analysable. When analysing other aged varnishes by HPLC–MS, it was also observed that the baseline was not straight, but showed some ''humps''. These two phenomena were seen earlier in the analysis of aged varnishes with capillary GC–MS [21]. Besides oxidation it is possible that condensation occurs during the ageing process. It is likely that the ''humps'' in the baseline are either caused by the presence of numerous oxidised species or the presence of a condensed fraction. Loss of the triterpenoid fraction and the formation of oligomers were demonstrated by size-exclusion chromatography (SEC) analysis of dammar films that were aged under xenon light [36]. Because HPLC is not suited for the analysis of large condensation products and it is not clear whether compounds with a high molecular mass may be difficult to detect by APCI-MS, other chromatographic and mass spectrometric techniques will be explored.

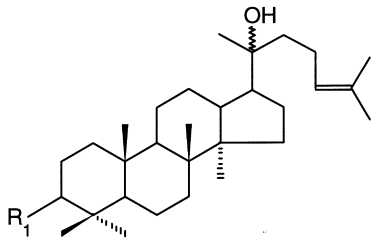
Since the amount of material that could be analysed by HPLC was very low in the case of aged varnishes, collection of the fractions and subsequent analysis with GC–MS was nearly impossible. Previous work by GC–MS already identified a number of constituents of these aged triterpenoid varnishes [21]. Most of the constituents separated by HPLC could be tentatively identified, as listed in Table 1, with this GC–MS information, the HPLC retention times, and the information obtained from the APCI-MS spectra, like the molecular mass information and the occur-



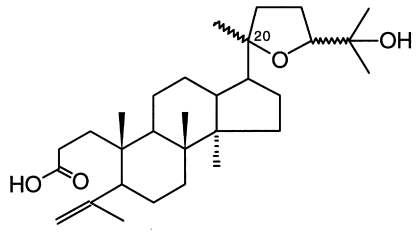
label	compound name	R_1
6	dammaradienol	OH
13	dammaradienone	O



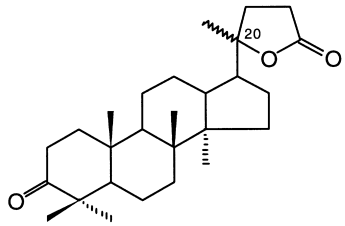
label	compound name
1	dammarenolic acid



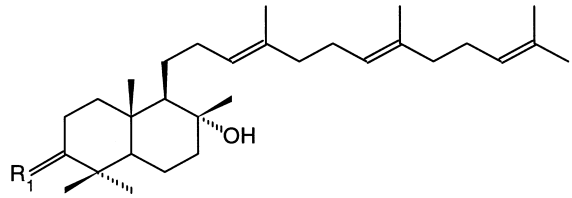
label	compound name	R_1
4, 5	hydroxydammarenone	O
8	dammarenediol	OH



label	compound name
26	20,24-Epoxy-25-hydroxy-3,4-seco-4(28)dammaren-3-oic acid



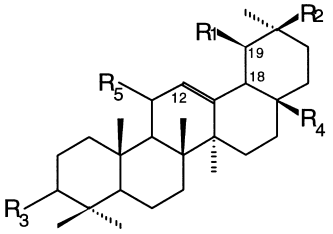
label	compound name
24	3-oxo-25,26,27-trinor-dammarane-24,20-lactone



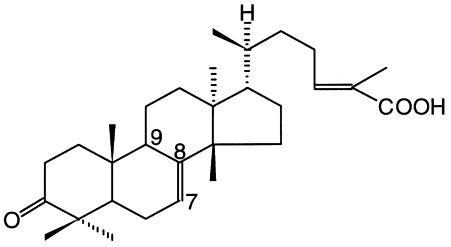
label	compound name	R_1
15	(3L,8R)-3,8-dihydroxypoly poda-13E,17E,21-triene	(H)OH
16	(8R)-3-oxo-8-hydroxypoly poda-13E,17E,21-triene	O

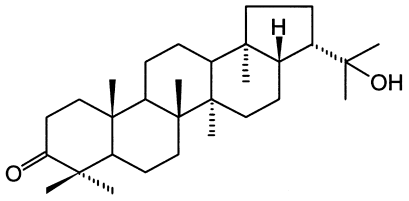
Fig. 3. Molecular structures of identified triterpenoid compounds (continued on next page).

rence of some useful fragment ions. Previous work by GC–MS identified ocotillone type molecules as the most abundant compounds of aged varnishes. Since ocotillone type molecules contain two epimeric centres (C20 and C24), it is likely that a number of stereoisomers are present. In contrast to the GC separation, these stereoisomers were well separated by HPLC. Molecules of the ocotillone type could be traced by HPLC–APCI-MS by applying mass chromatogram generation of their characteristic fragment ion of m/z 143, which corresponds to the oxidised side chain in ocotillone [21]. The fragmentation behaviour of an ocotillone type molecule (Fig. 9) will be described in Section 3.4. The identification of the specific isomers could not be achieved yet. Other oxidised stereoisomers with the dammarane skeleton (24 and 26) were also well resolved, whereas some isomeric compounds with the ursane/oleanane



label	compound name	R_1	R_2	R_3	R_4	R5
10	oleanolic aldehyde	H	CH_3	(H)OH	CHO	H_2
11	ursolic aldehyde	CH_3	H	(H)OH	CHO	H_2
7	oleanonic aldehyde	H	CH_3	O	CHO	H_2
9	ursonic aldehyde	CH_3	H	O	CHO	H_2
3	oleanonic acid	H	CH_3	O	COOH	H_2
2	ursonic acid	CH_3	H	O	COOH	H_2
21	11-oxo-oleanonic acid	H	CH_3	O	COOH	O
22	11-oxo-ursonic acid	CH_3	H	O	COOH	O
14	moronic acid[i]	H	CH_3	O	COOH	H_2





label	compound name	R_1
17	masticadienonic acid	O
18	isomasticadienonic acid[ii]	O
19	3-O-acetyl-2-epi-masticadienonic acid	CH_3COO
20	3-O-acetyl-2-epi-isomasticadienonic acid[ii]	CH_3COO

label	compound name
12	hydroxyhopanone

[i] In case of moronic acid, the double bond is located at C18.
[ii] In case of the iso compounds, the double bond is located at C8.

Fig. 3. (*continued*)

skeleton, such as 21/22 and 2/3/14, were not resolved by reversed-phase HPLC. Since these compounds are well separated by GC, the HPLC fractions could be identified by off-line HPLC–GC–MS. The aged varnish of Fig. 4b also contains two compounds which are indicative of the presence of an aged diterpenoid varnish (conifer resin), 15-hydroxy-7-oxodehydroabietic acid (15-OH-7-oxo-DHA) and 7-oxodehydroabietic acid (7-oxo-DHA) [37,38]. It is probable that residues of older varnish layers were present on the painting from which this sample was taken. The components with a relatively short retention time, particularly present in Fig. 4b, do not seem to be of triterpenoid origin and were not identified yet. Other unidentified peaks with longer retention times of Fig. 4 were probably too polar to be analysed by GC. These compounds could not be identified solely on the basis of their APCI-MS

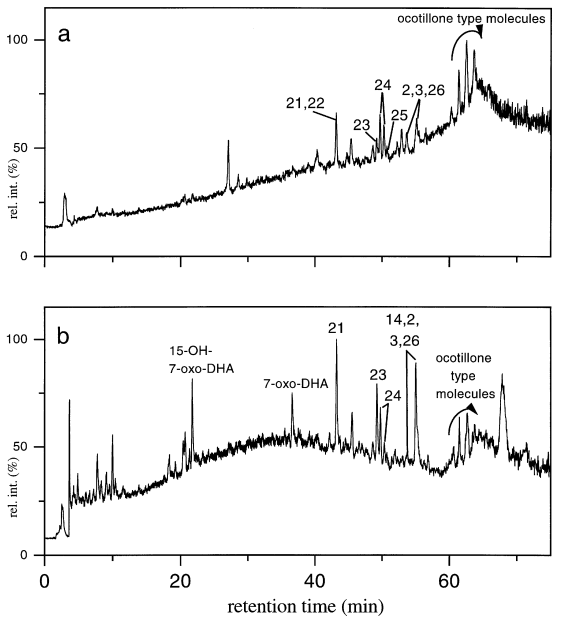


Fig. 4. Total ion current traces of reversed-phase HPLC–APCI-MS, using the positive ion mode, of aged dammar varnish (a) and aged mastic(/diterpenoid) varnish (b) (peak labels refer to Table 1).

spectra. The specific information that was obtained from the APCI-MS spectra of triterpenoids will be in Section 3.4.

3.3. Discrimination between aged dammar and mastic varnish by HPLC–APCI-MS

GC–MS is often used to distinguish between aged dammar and mastic varnishes. Moronic acid, which is only present in mastic resin, can be used as a marker to discriminate between aged dammar and mastic varnishes [39], and is easily separated from the other components by GC–MS [21]. Under the HPLC conditions that were used here, moronic acid (14) co-elutes with two of its isomers, oleanonic (3) and ursonic acid (2), which show similar mass spectra under APCI conditions as moronic acid. The inability to separate these isomers excludes discrimination between dammar and mastic varnish on the basis of the occurrence of moronic acid. However, other molecular markers may serve this goal. Since fresh dammar resin contains both compounds with the oleanane and ursane skeleton, whereas fresh mastic contains only the oleanane skeleton, [39], this fact can also be used to make the distinction between the two types of varnishes. Two specific oxidised isomeric compounds, with either an oleanane or ursane skeleton, which could not be identified yet by their EI spectra, are often found in aged varnishes [21]. Under APCI conditions these molecules are recognised by the protonated molecule, represented by a peak at m/z 469, and the characteristic fragment ion of m/z 233 (23 and 25). The two isomers with the oleanane and ursane skeleton are well resolved by HPLC–APCI-MS using acetonitrile and water as eluents and can be traced by applying mass chromatogram generation. Fig. 5 shows that an aged

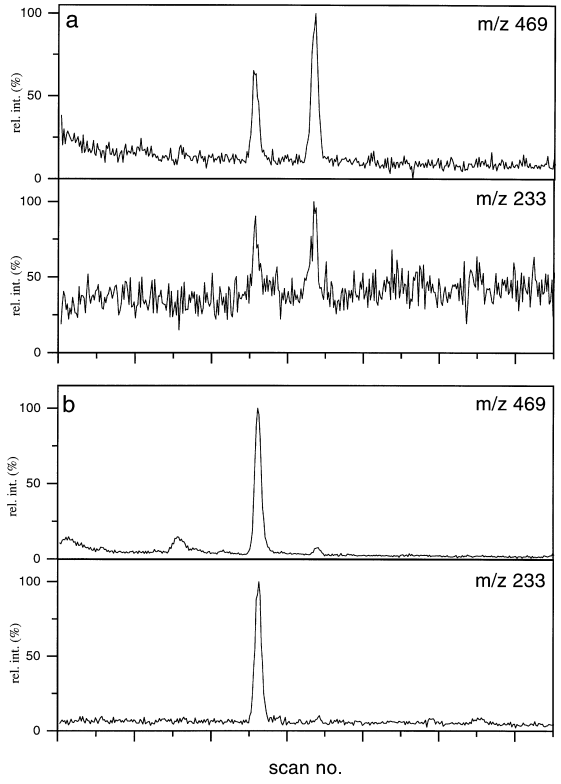


Fig. 5. Selected ion current profiles at m/z 469 and m/z 233 during the HPLC–APCI-MS analysis of aged dammar varnish (a) and aged mastic varnish (b).

dammar varnish (a) contains both isomers, whereas an aged mastic varnish (b) contains only the compound with the oleanane skeleton. HPLC–APCI-MS can thus be used for the discrimination between aged dammar and mastic varnish. There are other notable differences between the TICs of aged dammar and mastic varnish, which are shown in Fig. 4. However, these differences are probably due to other factors, for example the number of years the varnishes have aged on the paintings, the environmental conditions, or the possible additions by painting restorers.

3.4. Ionization and fragmentation behaviour under APCI-MS conditions

HPLC–APCI-MS has been reported to be useful for the purpose of structural elucidation [22,28,29]. The mass spectrometric behaviour of triterpenoids under HPLC–APCI-MS conditions was studied, using an acetonitrile–water mixture as the mobile phase. The effect of instrumental parameters, like cone voltage, probe temperature, corona voltage and source temperature on the appearance of the mass spectra was investigated in the positive ion mode, by on-flow injection of oleanolic acid in acetonitrile–water (90:10). Only the cone voltage was observed to have an effect on the fragmentation patterns. As expected and illustrated in Fig. 6a–c, increasing the cone voltage led to more fragmentation of the protonated molecule via collision induced dissociation. The mass spectrometric behaviour of oleanolic acid under APCI conditions in the negative ion mode was also investigated (Fig. 6d). In our experiments, the ion yield in the negative ion mode is approximately a factor 20 lower compared to that in the positive ion mode. This relatively low probability of detecting a negatively charged ion under the high-pressure conditions in APCI can be explained by the readily detachment of an electron, when colliding with other species. A high electron affinity of the analyte will increase this probability of detection. When a compound is detected in the negative ion mode, it can be concluded that its electron affinity is relatively high. Whereas functional groups were easily lost when the molecule was protonated in the positive ion mode (Fig. 6a–c), this phenomenon was not seen in the negative ion mode. Molecules were deprotonated without any further fragmentation.

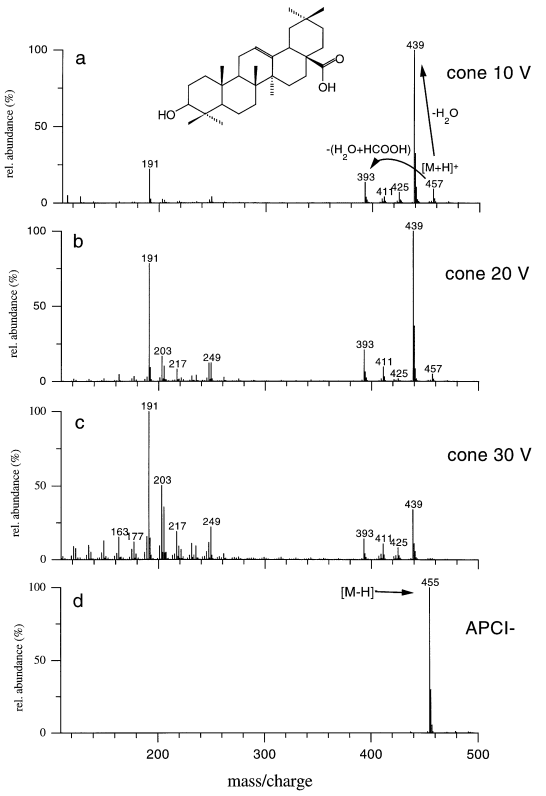


Fig. 6. Mass spectra obtained by HPLC–APCI-MS demonstrating the effect of the cone voltage in the positive ion mode (a–c), and the mass spectrum obtained in the negative ion mode (d) of oleanolic acid.

Especially in the case of molecules with an acidic group or an hydroxyl group, spectra obtained in the positive and negative ion mode are complementary. Molecular mass information is obtained in the negative ion mode, whereas information on the presence of functional groups is gained by analysis in the positive ion mode.

The fragmentation behaviour under APCI-MS conditions and EI conditions (70 eV) is compared for three molecules with the dammarane or the oleanane skeleton. The mass spectra of hydroxydammarenone, a molecule with the dammarane skeleton, obtained by EI (70 eV) and HPLC–APCI-MS are shown in Fig. 7. The MS–MS results of hydroxydammarenone will be described in Section 3.5. Complete elimination of the hydroxyl group at C20 as H_2O is observed under EI conditions (Fig. 8). The side chain of the dammarane skeleton is cleaved at C(17)

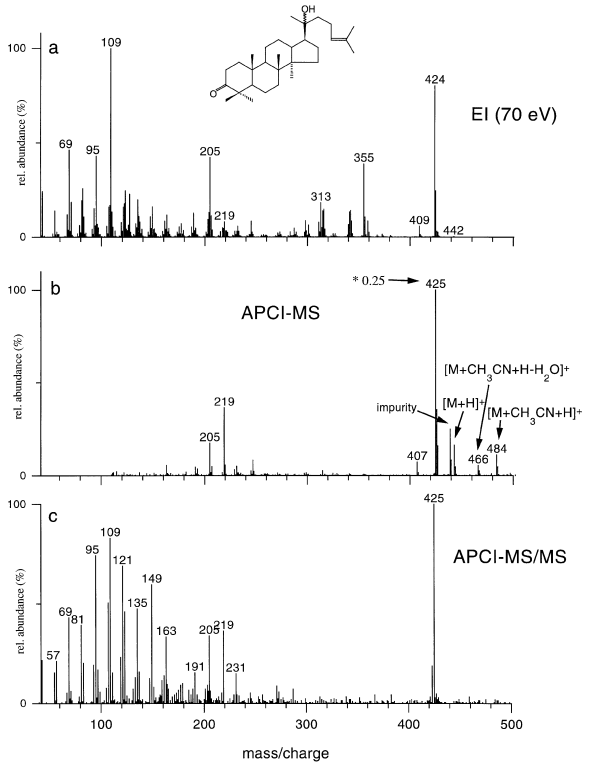


Fig. 7. Mass spectra obtained by EI (70 eV) (a), HPLC–APCI-MS (b), and HPLC–APCI-MS–MS (c) of hydroxydammarenone. ['*0.25' in (b) means an attenuation of the peak intensity at m/z 425 by a factor of four].

(m/z 355) and ring C cleavage with concerted hydrogen transfer of the dammarane skeleton produces the fragment ion which is represented by a peak at m/z 205 [34]. Under APCI-MS conditions (Fig. 7b, Fig. 8), the protonated molecule eliminates the hydroxyl group at C20 very easily. The presence of m/z 407 suggests that another molecule of water is lost possibly via the keto substituent. This is in accordance with the findings of Harrison [40], who states that ketones show some elimination of water under chemical ionisation conditions using ammonia. The presence of acetonitrile adducts (represented by peaks at m/z 484 and m/z 466) assists in the molecular mass determination. As in EI, cleavage of ring C occurs, resulting in both fragment ion peaks at m/z 219 and m/z 205. The APCI-MS spectra of other molecules with the dammarane skeleton (Table 1) showed similar results. Cleavage of ring C, with the exception of compounds 24 and 26, and loss of hydroxyl, aldehyde and keto groups are mainly observed. Compounds 1 and 13 showed some fragment ions which could not be identified yet. Fig. 9 shows the EI and APCI-MS spectra of one of the ocotillone type stereoisomers. The configuration at C20 and C24 of the ocotillone type molecule could not be determined yet. Under EI conditions, cleavage of ring C with concerted hydrogen transfer occurs, which results in a fragment ion peak at m/z 205 (Fig.

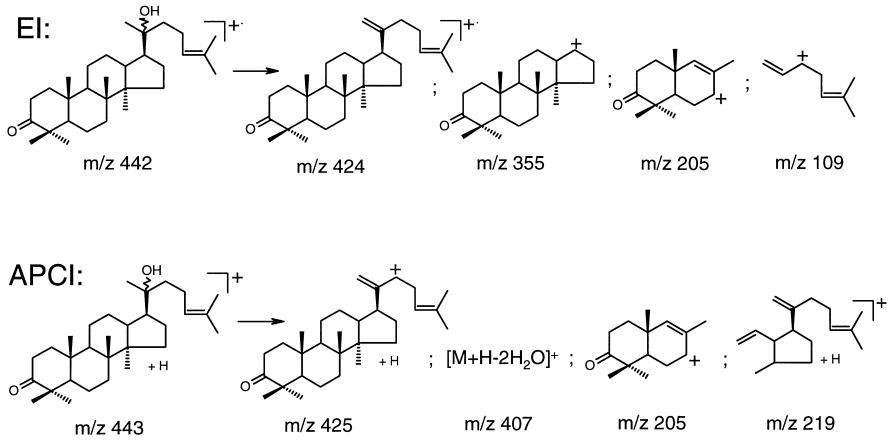


Fig. 8. Proposed principal mass spectral fragments of hydroxydammarenone under EI and APCI conditions.

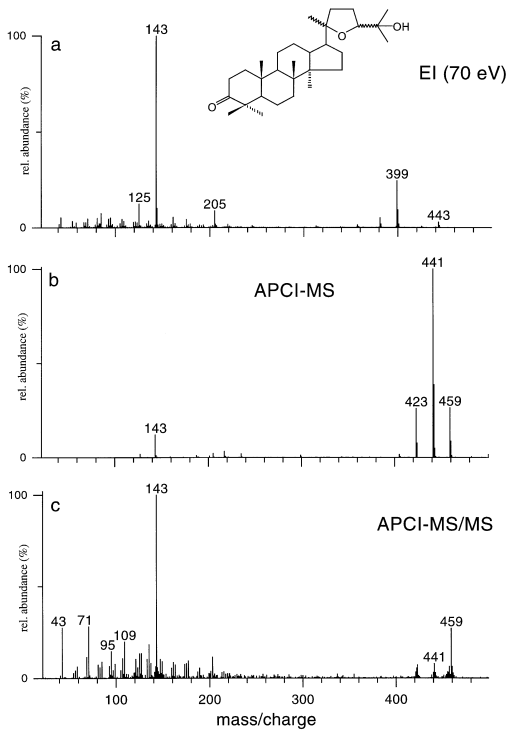


Fig. 9. Mass spectra obtained by EI (70 eV) (a), HPLC–APCI-MS (b), and HPLC–APCI-MS–MS (c) of an ocotillone type molecule.

10). The side chain is cleaved at two locations, which produces the fragment ion peak at m/z 399 and the base peak at m/z 143. In addition to water elimination, the fragment ion peak at m/z 143 is also present in the APCI-MS spectrum and is therefore indicative of the presence of this hydroxyisopropylmethyltetrahydrofuran side chain.

The mass spectra of oleanonic aldehyde obtained by EI (70 eV) and HPLC–APCI-MS are shown in Fig. 11. Under EI conditions, the aldehyde substituent is eliminated to a certain extent (Fig. 12). A typical retro-Diels–Alder (rDA) rearrangement takes place, producing peaks at m/z 232 and m/z 203 [30,31]. Fig. 11b shows that the protonated molecule obtained under APCI-MS conditions is relatively stable. In addition to adduct formation with acetonitrile (represented by a peak at m/z 480), some elimination of water occurs from $[M+H]^+$ to generate the species represented by a peak at m/z 421, due to the presence of the keto or the aldehyde substituent. Elimination of 28 u from $[M+H]^+$ (to generate a species represented by a peak at m/z 411) can best be explained by the loss of CO from the aldehyde substituent. This loss is also observed in HPLC–APCI-MS analysis of phenolic compounds which bear an aldehyde substituent [26]. According to Madhusudanan et al. [41], the rDA rearrangement takes place under chemical ionisation (CI) conditions and this leads to ions corresponding to both the diene part and the dienophile part of the molecule (Fig. 12). This is unlike the EI spectra where rDA reaction gives only the diene ion. In the case of APCI it seems somewhat more complex. In Fig. 11b there are a number of peaks present around m/z 200. Other molecules with an oleanane or the isomeric ursane

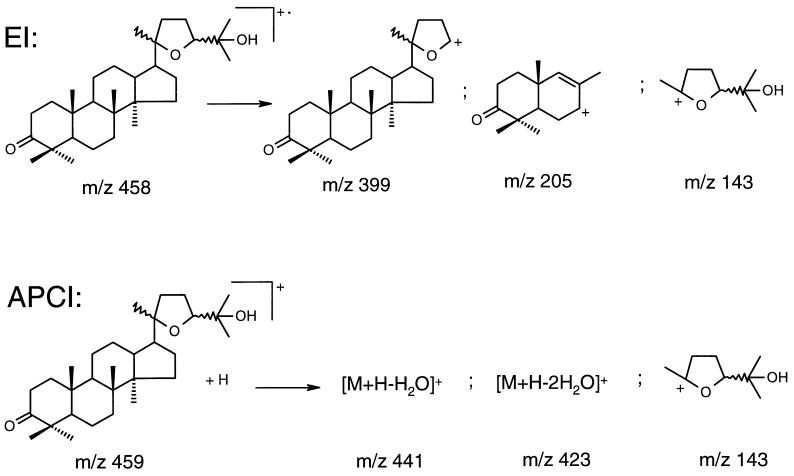


Fig. 10. Proposed principal mass spectral fragments of an ocotillone type molecule under EI and APCI conditions.

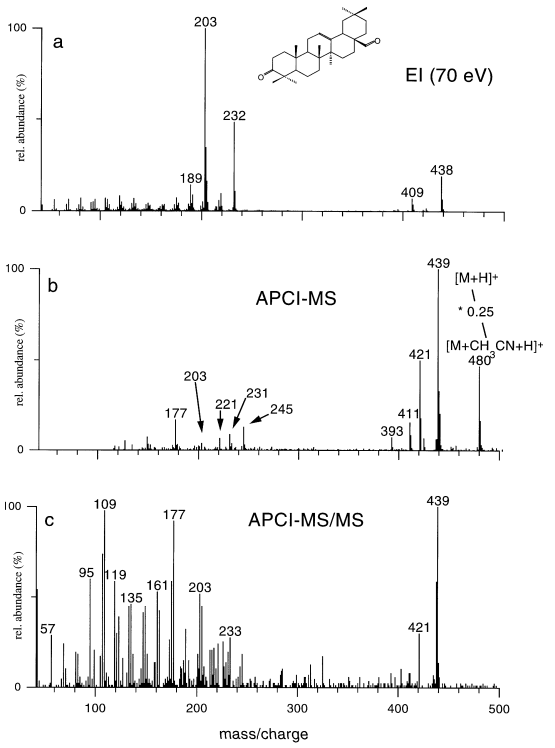


Fig. 11. Mass spectra obtained by EI (70 eV) (a), HPLC–APCI-MS (b), and HPLC–APCI-MS–MS (c) of oleanonic aldehyde. ['*0.25' in (b) means an attenuation of the peak intensities at m/z 480 and m/z 439 by a factor of four].

skeleton, such as oleanolic acid (Fig. 6c), show similar fragment ion peaks in this mass range which are not seen when these molecules are analysed under EI conditions. The presence of fragment ion peaks in this mass range can best be explained by double bond migration from C12 to another location in the molecule. When a molecule is protonated under APCI conditions, this proton can be positioned on a double bond since this feature has a relatively high proton affinity [40]. It is likely that protonation of the double bond under APCI conditions facilitates the migration. According to Budzikiewicz et al. [30], bond migration in the molecular ion seems to occur occasionally under EI conditions. The migration of double bonds has also been found to occur in acyl lipids containing unsaturated fatty acids during the CID processes in low energy MS–MS experiments [42]. When the HPLC–APCI-MS experiments were repeated, it was observed that the distribution of the smaller fragment ion peaks did not reproduce very well in contrast to the molecular ion region. The same fragment ion peaks were observed, but their intensity ratios differed. The ion life time prior to mass analysis is relatively long in HPLC–APCI-MS experiments. In addition, the gas phase involved is relatively dense, which results in multiple collisions [27]. It is likely that these factors are responsible for the poor reproducibility of fragment ion peaks under APCI conditions. Furthermore, since a HPLC gradient is used, the atmospheric conditions change continuously, which is very likely to have an effect on the fragmentation behaviour of the analytes. The condition of the HPLC column changes due to its age and usage history, and this may have an effect on the retention times of analytes and consequently on the corresponding APCI conditions.

Other compounds with the oleanane or ursane skeleton (Table 1) showed a similar behaviour under APCI-MS conditions. In addition to hydroxy, aldehyde and keto groups, the carboxylic acid substituents (in the 17 position) were eliminated to a certain extent as formic acid (2, 3, 14, 21 and 22). The loss of formic acid is energetically more favourable than the loss of water and carbon monoxide together [43]. However, carboxylic acid groups on other positions, present on triterpenoids with other skeleton types (1, 17, 18, 19, 20 and 26), were not split off under APCI conditions. Fragment ions represented by peaks around m/z 200, which were probably induced by double bond migration, were observed, but these fragment ion peaks did not reproduce well and were of low intensity. A peak at m/z 233 was found to be present in both the EI and APCI-MS spectra of compounds 23 and 25 (mass spectra not shown), and, as was mentioned before, this fragment ion peak was used to discriminate between aged dammar and mastic varnishes. When a hydroxyl group was present at C3 (10 and 11), water was eliminated and fragment ions of m/z 205 and m/z 219 were found, which were probably formed in a rDA reaction.

Some triterpenoids with the euphane and hopane skeleton type (12, 18 and 19) showed some fragment ion peaks, at m/z 179, 127, 125, 247 and 191, which could not be identified yet. Under EI conditions, a fragment ion peak at m/z 127 is found to be indicative of methyl masticadienonate [19]. This

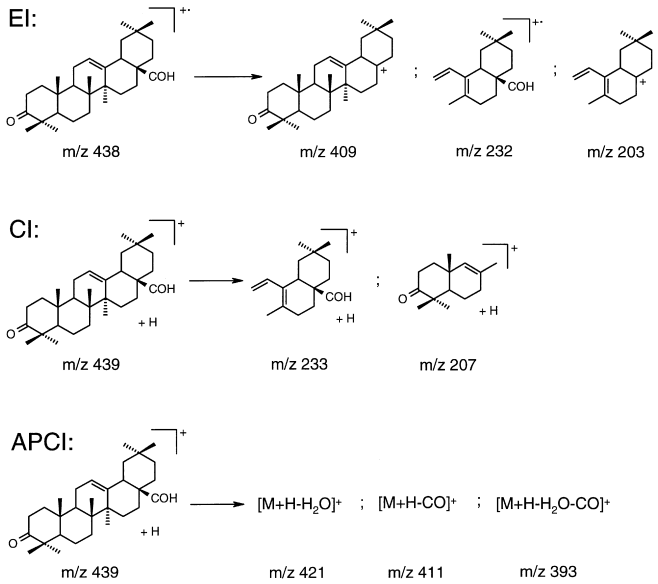


Fig. 12. Proposed principal mass spectral fragments of oleanonic aldehyde under EI, CI and APCI conditions.

useful fragment ion was also formed under APCI conditions. The mass spectra of the bicyclic triterpenoids were straightforward in that the functional groups were lost (15 and 16). In case of compound 16, the long side chain was cleaved producing fragment ion peaks at m/z 219 and m/z 205. The additional fragment ion of m/z 219 could not be identified yet.

3.5. Investigation of the fragmentation of triterpenoids under APCI–MS–MS conditions

Low-energy HPLC–APCI-MS–MS spectra were obtained with a triple quadrupole mass spectrometer, using argon as the collision gas. Fig. 7c depicts the APCI-MS–MS spectrum of an ion of 425 u, which is formed by protonation of hydroxydammarenone followed by the loss of a water molecule. Fig. 9c and Fig. 11(c) show the APCI-MS–MS spectra of ions of 459 u and of 439 u, which correspond to a protonated ocotillone type molecule and protonated oleanonic aldehyde, respectively. All MS–MS spectra show peaks representing ring cleavage fragment ions which indicate that molecules of triterpenoid origin were analysed [44]. The ocotillone type molecule also produces the characteristic fragment ion of m/z 143 under MS–MS conditions. In addition to the elimination of functional groups, mainly non-specific fragment ion peaks below m/z 240 are produced, as seen in Fig. 7c, Fig. 9c and Fig. 11c. When the pressure of the collision gas, argon, is relatively low, fragmentation of the selected protonated molecule is minimal leading to few ring cleavage fragment ions. When the pressure was increased, fragmentation again only produced small fragment ion peaks but now with a higher intensity. Unfortunately, these fragment ion peaks observed were similar for triterpenoids with dammarane, oleanane and ursane skeletons, which are often isomeric compounds, and are therefore of little diagnostic value for the molecular identification of these structures. In most cases, as in Fig. 7c and Fig. 11c, the MS–MS spectra did not resemble the APCI-MS spectrum nor the EI spectrum. The fragment ions represented by peaks in the MS–MS spectra (produced in a collision cell) and even those in the APCI-MS spectra (high pressure in the source) are generated by CID processes. Ions that are represented by peaks in the APCI-MS spectra have reached the detector and can be considered as relative ‘‘cool’’ and stable, because unstable ‘‘hotter’’ ions are likely to be destroyed by the multiple

collisions in the dense gas phase of the ion source. In the APCI-MS spectra of triterpenoids mostly peaks representing protonated molecules (MH^+) were observed. In the process of MS–MS analysis of these ions, the internal energy is increased in a short time frame. The unimolecular dissociation of these excited MH^+ ions apparently leads preferentially to ring cleavage ions because peaks representing C-ring rearrangement ions have a very low relative intensity in the spectra.

Hazai et al. [45] identified pentacyclic triterpenes by GC–MS–MS using a triple quadrupole MS system and thus low energy collisions. In contrast to our observations, the collision-activated dissociation spectra were found to be similar to EI spectra [45]. It can be concluded that when these triterpenoid molecules are ionised by EI, both low energy and high energy collisions give rise to the same fragmentation behaviour. The major difference between these GC–MS–MS experiments and our HPLC–APCI-MS–MS experiments (using the positive ion mode) is the fact that in the latter case the molecules are ionised by protonation (CI). As stated by Harrison [40], the fragmentation mode of the even-electron protonated molecules is quite different from the fragmentation modes of the odd-electron molecular ions formed by EI ionisation. Since the MS–MS spectra of Fig. 7c and Fig. 11c do not resemble the corresponding EI spectra (Fig. 7a, Fig. 11a) protonation of a triterpenoid molecule is likely to be an important factor determining the fragmentation behaviour under MS–MS conditions. Retro-Diels–Alder rearrangement in the 12-unsaturated oleanane or ursane type compounds (Fig. 11) as well as ring C cleavage of dammarane type compounds (Fig. 7) are observed to occur to a smaller extent when the analytes are protonated.

3.6. Concluding remarks on the applicability of HPLC–APCI-MS(–MS) in the study of triterpenoids

APCI-MS is found to be a mild ionisation method, in which predominantly protonated molecules are formed. Loss of water is observed to a large extent for molecules which contain a hydroxyl group and to a much smaller extent for molecules with a keto group. Triterpenoids with an aldehyde substituent show some loss of CO. The dammarane skeleton type molecules, which have a saturated ring system, predominantly show cleavage of ring C, similar to EI. The fragmentation of the 12-unsaturated oleanane/ursane skeleton type molecules is more complex, probably because of double bond migration. This latter phenomenon prevents the identification of the location of the double bond and it complicates the interpretation of the spectra. Under negative ionisation conditions deprotonated molecules are formed without any other fragmentation, which gives molecular mass information. In conclusion, in addition to molecular mass information, APCI-MS spectra provide extra information about some frequently occurring functional groups in triterpenoids.

Additional MS–MS produces fragment ion peak patterns which are characteristic for molecules of the triterpenoid class. Unfortunately, identification of specific triterpenoids is not possible, since the MS–MS fragment ion peaks are the same for a number of different triterpenoids. HPLC–APCI-MS–MS of the aged triterpenoid varnishes provided information about the functional groups present, but this information is already available in the APCI-MS spectra.

In addition to the MS data, the retention times and the possibility of comparison with UV–Vis absorption data will help in the determination of the constituents of complex samples. Most constituents of both fresh triterpenoid resins and aged varnishes are well resolved by reversed-phase HPLC using acetonitrile and water as eluents. The separation of two isomers with an oleanane and ursane skeleton enables the discrimination between aged dammar and mastic varnish. In contrast to the fresh resins, only a small part of the aged varnishes is analysable by HPLC–APCI-MS. This is probably due to the presence of a condensed fraction, which HPLC analysis is less suited for.

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