Application of HPLC-PBMS to the Identification of Unknown Components in a Triterpenoid Fraction of *Arbutus unedo* Fruits

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Key Words:

HPLC-MS
Particle beam
HRGC-MS
***Arbutus unedo* L.**
Triterpenoids
Allelopathy

Summary

An extract fraction from fruits of *Arbutus unedo*, L., was cleaned-up by column chromatography and shown by NMR to be a mixture of isomers that resists further attempts at separation by conventional chromatographic methods. High resolution gas chromatography-mass spectrometry (HRGC-MS) confirms the presence of triterpenoid isomers but does not allow separation of all the components. This can be improved by trimethylsilylation but the absence of molecular ions and the complex spectra are difficult to interpret. Complete separation can be achieved by high pressure liquid chromatography (HPLC) coupled to a mass spectrometer by means of a particle beam interface (HPLC-PBMS). Four triterpene compounds are identified through analysis of the corresponding mass spectra: α-amyrin, β-amyrin, and lupeol, have for the first time been identified in *Arbutus unedo*, L. fruits. A new natural triterpene tentatively identified as olean-12-en-3β,23-diol is described for the first time.

1 Introduction

The strawberry tree (*Arbutus unedo*, L.) is a spontaneous Mediterranean species that grows in mountainous regions. The strawberry like fruits have some importance in local rural economies that use the berries for production of a highly appreciated alcoholic beverage. Leaves and fruits are used in traditional folk medicine to prepare diuretic and urinary antiseptic potions. More recently indications of some allelopathic activity towards surrounding species were reported. It is a popular believe that the excessive consumption of the fruits produces some kind of addiction with alcoholic-like syndromes.

Oleane and lupane triterpenes have been reported in *Melitotus* sp. as active secondary metabolites in germination of test species [1]. The search for active secondary metabolites in the *A. unedo*, L. fruits afforded a triterpenoid fraction containing a mixture of triterpene isomers, which resisted successful separation by conventional chromatographic procedures. HRGC methods failed to separate the isomers and HRGC-MS afforded only ambiguous data. Successful separation was achieved by HPLC and tentative identification was carried out on the basis of the mass spectra of underivatized compounds obtained by coupling HPLC to a mass spectrometer through a particle beam interface (HPLC-PBMS). β-Sitosterol, β-amyrin, α-amyrin, and lupeol could be identified by comparison of chromatographic and mass spectral data with authentic samples. HPLC-PBMS allowed the detection of a new oleanane-type triterpene for which the structure of olean-12-en-3β,23-diol is proposed on the basis of HPLC-PBMS and NMR data.

2 Materials and Methods

2.1 Instrumentation

Gas chromatography was performed with a Carlo Erba instrument of the Vega series, model 5300 equipped with a split-splitless injector, a flame ionization detector, and a home-made 25 m × 0.25 m .d. silica capillary column coated with OV-101, d_f = 0.25 μm. The oven temperature was linearly programmed from 250 to 300 °C at 2.5 °min^{-1}. Injector and detector temperatures were 300 °C. Hydrogen (P_i = 70 kPa) was used as carrier gas. Peak integration was performed with a Shimadzu CR 3A computing integrator. HRGC-MS measurements were made under identical chromatographic conditions with a Shimadzu instrument, model QP-1000, under the following conditions: ion source temperature 250 °C, direct interface temperature 325 °C, scan rate 1.2 s/decade from *m/z* 50 to *m/z* 500. HPLC was performed with a Gilson instrument equipped with a 250 × 4 mm i.d. column packed with 5 μm Lichrosorb RP Select B (E. Merck, Darmstadt, Germany). Elution was carried out with 0.1% acetic acid/methanol:water (96:4), flow rate 0.7 ml min^{-1}, with light-scattering detection (LSD) using a light-scattering detector from Sedere (Alfortville, France), model Sedex 55, operated at gain 8, temperature 62 °C, and air as nebulization gas at pressure of 250 kPa. HPLC-PBMS runs were performed under identical chromatographic conditions with a VG Trio-1000 instrument equipped with a particle-beam (PB) interface. EI spectra were obtained at 70 eV. ^{1}H-NMR and ^{13}C-NMR analysis were performed with Bruker ARX 400 MHz instrument using $CDCl_3$ as solvent.

2.2 Chemicals

All solvents were p.a. grade from E. Merck (Darmstadt, Germany). Solvents used in HPLC measurements were HPLC grade, Lichrosolv from E. Merck (Darmstadt, Germany). Water was home-purified with a Milli-Q system (Millipore, Bedford, MA, USA). Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were from Fluka. Pyridine was p.a. from E. Merck (Darmstadt, Germany) and was dried over sodium hydroxide.

2.3 Extraction

Finely mixed, lyophilized *Arbutus unedo* fruits (250 g) were left in acetone-water (7:3, 4 L) in the dark for 48 h. After filtration, the solvent was removed by rotary evaporation at a temperature not exceeding 50 °C. The residue was dissolved in the minimum amount of water and extracted with diethyl ether (2.8 L). The

ethereal layer was washed with water, dried over magnesium sulfate, and the solvent removed by rotary evaporation, yielding 5.13 g of dry residue. The residue (4 g) was eluted from a 70–230 mesh silica-gel (300g) glass column (90 × 3 cm) with approximately 1 L *n*-hexane:toluene. Fractions of approximately 5 mL were collected and submitted to thin-layer chromatography for homogeneity testing. Identical fractions were united in a single batch. Three different fractions were obtained from the chromatographic run. The main fraction (~1 g) contained the triterpenoid compounds and was further submitted to separation on a neutral alumina column by elution with diethyl ether. Fractions of approximately 5 mL were collected and submitted to thin-layer chromatography for homogeneity testing. Two new fractions A (700 mg) and B (β-sitosterol, 60 mg) were collected and concentrated to a minimal volume on a rotary evaporator.

2.4 Trimethylsilylation

A sample of fraction A (~1 mg) was transferred into a screw-cap glass vial and the solvent evaporated under a light stream of nitrogen. The residue was dissolved in 100 µL of dry pyridine, 500 µL HMDS, and 10 µL of TMCS. The solution was heated at 50 °C for 1 h. After centrifugation, a 0.2 µL aliquot of the supernatant was used for chromatography.

3 Results and Discussion

Literature is sparse on references to natural products in *Arbutus unedo*, L. Uses and popular beliefs associated to the berries together with potential allelopathic activity motivate the search for new biologically active compounds and environmentally friendly herbicides.

Extracts from the fruits were submitted to preliminary screening by fractionation in solubility groups. The ether-soluble components from an acetone:water extract afforded two chromatographic fractions of apparent simplicity. The latter eluting fraction (fraction B in experimental) was a pure compound identified as the ubiquitous β-sitosterol by chromatographic (HRGC) and spectroscopic (^{1}H-NMR, ^{13}C-NMR, and MS) comparison with an authentic sample. **Figure 1** shows the HRGC-MS chromatogram of fraction A. The first peak was identified as β-amyrin on the basis of its mass spectra (**Table 1**). The other components cannot be separated and HRGC-MS deconvolution was not convincing. The recorded mass spectra were ambiguous and only gave an indication of the nature of the compounds as a mixture of functionalized lupane and oleanane derivatives. This was confirmed by ^{13}C-NMR data obtained from the mixture.

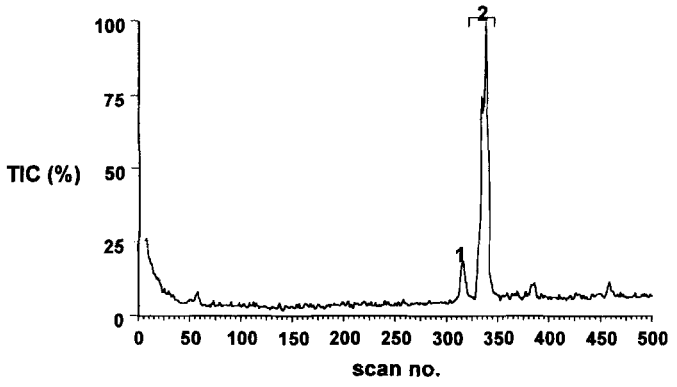


Figure 1. TIC obtained from HRGC-MS assay of fraction A (see experimental) of the neutral extract of the fruits of strawberry tree (*Arbutus unedo*, L.). Peaks: 1) β-amyrin; 2) mixture of α-amyrin, lupeol, and a third unknown component. Conditions as in the experimental part.

Table 1. Mass spectra [4] of the compounds identified by HPLC-PBMS in the neutral fraction of the acetone:water extract of the fruits of *Arbutus unedo*, L.

Peak no.	Compound	*m/z* (%)
1	β-Amyrin	426 (M^+, 9), 411 (3), 408 (19), 218 (100), 207 (41), 205 (5), 203 (29)
2	α-Amyrin	426 (M^+, 19), 408 (15), 218 (100), 207 (21), 205 (6), 203 (24), 189 (29)
3	Lupeol	426 (M^+, 15), 408 (15), 297 (14), 229 (8), 218 (35), 207 (56), 205 (11), 189 (44)
4	Olean-12-en-3β,23-diol (**1**)	442 (M^+, 0.3) 424 (M^+–18, <1)), 409 (M^+–15–18, 2), 408 (2), 229 (7), 223 (<1%), 218 (88), 205 (21.6), 203 (100), 189 (29)

Trimethylsilylation of the mixture revealed the existence of a new peak concealed under the unresolved region in Figure 1, with an increased retention time (peak 4 in **Figure 2**). The mass spectral data obtained from HRGC-MS runs confirmed the presence of β-amyrin (peak 1). Mass spectral analogies and comparison with authentic samples allowed identification of α-amyrin and lupeol (peaks 2 and 3). The TMS derivative of peak 4 suggested the presence of a dihydroxylated oleanane or lupane.

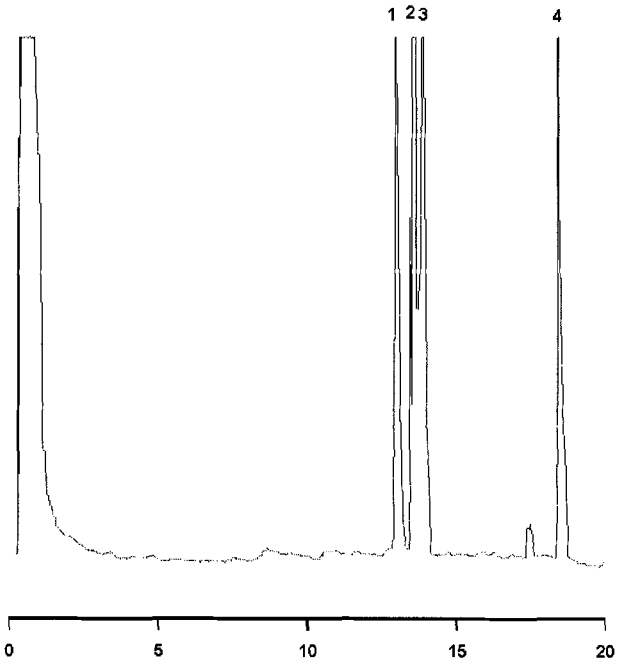


Figure 2. Chromatogram obtained from HRGC assay of trimethylsilylated fraction A (see experimental) of the neutral extract of the fruits of strawberry tree (*Arbutus unedo*, L.). Peaks: 1) β-amyrin; 2) β-amyrin; 3) lupeol; 4) olean-12-en-3β,23-diol. The identity of the peaks was established through HPLC-PBMS experiments. Conditions as in the experimental part.

However, the complexity of the spectra and the absence of a clear cut molecular ion did not permit further conclusions.

The selectivity of HPLC methods has been used for the of triterpenoid compounds, with a success not hitherto obtained by HRGC or by classical chromatographic methods [2,3]. On the other hand, mass spectrometry constitutes an extremely useful tool for structural elucidation in the triterpene field [4,5]. In general, the presence of a nuclear double bond controls the fragmentation behaviour, and characteristic mass spectral features allows assignment of a given triterpene to one of the major classes [4]. The location of eventual functional groups can be narrowed by consideration of the fragmentation pattern [4,5]. Good HPLC separation of all the components of fraction A was obtained under isocratic conditions with 0.1% acetic acid in methanol:water (94:6). Detection of all the components present in the sample was achieved with an evaporative light-scattering detector (ELSD). This is an universal detector that responds to mass of eluted compounds, is independent of any other property of the analytes, and is insensitive to changes in eluent flow or composition [6,7]. Detection is based on the quantity and size of droplets formed in a nebulizing chamber, after evaporation of the solvent [8]. The analogy of the operating principles makes ELSD very useful for the setting up operating conditions for the particle beam interface and for the control of the HPLC-PBMS operation.

Figure 3 shows the total ion current trace (TIC) for the HPLC-PBMS run. All mass spectra presented a molecular ion and characteristic fragmentation patterns. On the basis of mass spectral data (Table 1) the compounds were identified as the pentacyclic triterpenoids β-amyrin (peaks 1), α-amyrin (peak 2), and lupeol (peak 3). ^{13}C-NMR spectral analysis of the mixture gave data coincident with those described by Bhattacharyya *et al.* [9].

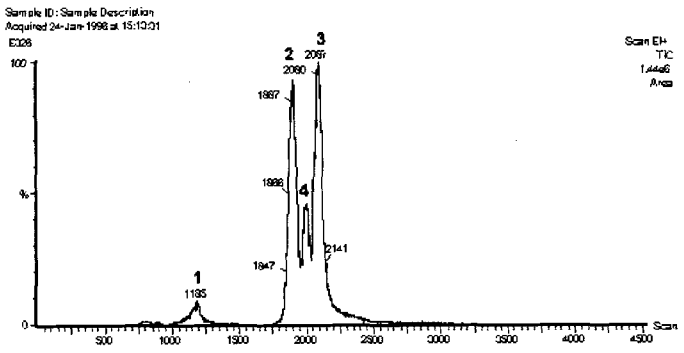
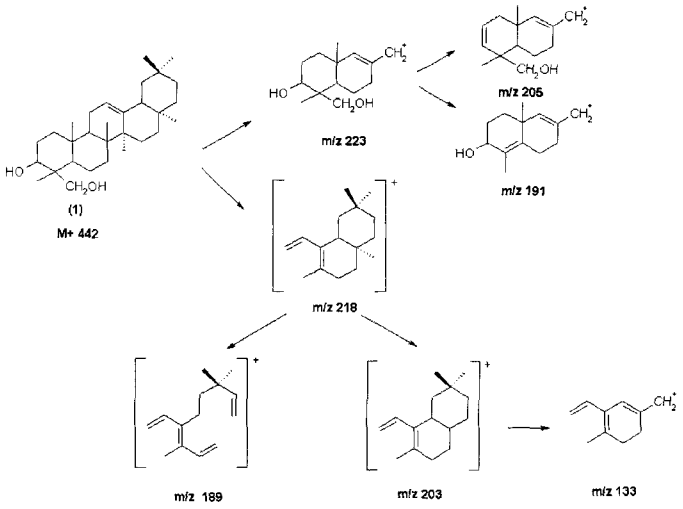


Figure 3. Chromatogram obtained from HPLC-PBMS assay of fraction A (see experimental) of the neutral extract of the fruits of strawberry tree (*Arbutus unedo*, L.). Peaks: 1) β-amyrin; 2) α-amyrin; 4) olean-12-en-3β,23-diol; 3) lupeol. Conditions as in the experimental part.

The most significant diagnostic ions for each compound may be used for identification of the components by mass chromatography.

The EI mass spectrum of peak 4 as obtained in the HPLC-PBMS assay, presents a characteristic fragmentation pattern [4,5]. Diagnostic ions are: *m/z* 424 ($M^+ - 18$), the fragments arising from D/E rings at *m/z* 229, 218, 203, and 189, and from A/B rings at *m/z* 223 $[C_{14}H_{23}O_2]^+$ and *m/z* 205 $[223 - H_2O]^+$ (**Scheme 1**).

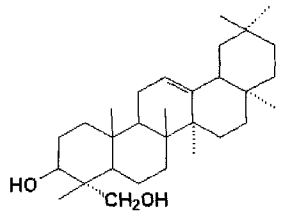


Scheme 1

These data are in agreement with the presence of a hydroxymethyl group at either C-23 or C-24 [10]. Also the HRGC-MS mass spectra of the trimethylsilyl derivative showed prominent *m/z* 483 $[M - TMS - 31]^+$ and *m/z* 265 $[223 + TMS - 31]^+$. This suggests the location of the hydroxymethyl group at an angular position [11] and is in agreement with ^{1}HNMR and ^{13}CNMR data reported for analogues [10,12,13]: the methine proton at 3.49 (s, large) is characteristic of triterpenes with a C-3 equatorial hydroxyl group; The ^{1}HNMR signals at δ 3.20 (s, broad) and δ 3.66 (s, broad) and the ^{13}CNMR signal δ 71.7 completely agree with the presence of a C-23 hydroxyl group [10, 12].

The chromatographic behavior is also that expected for a *trans*-3,4 stereochemistry. Being a diol, this compound should not be difficult to distinguish chromatographically and to separate from its isomers in apolar solvents. This is prevented by the intramolecular hydrogen bonding, only possible through the coplanarity of both substituents. The use of an acidic protic solvent in HPLC disrupts the hydrogen bond, thus permitting the separation.

On the basis of these data the compound corresponding to peak 4 is tentatively identified as olean-12-en-3β,23-diol (**1**). This is the first report of this compound as a new natural product.



(1)

4 Conclusion

Lupeol, α-amyrin, β-amyrin and a new natural compound tentatively assigned as olean-12-en-3β,23-diol were identified in a neutral extract of *Arbutus unedo*, L. fruits through the combined use of HRGC-MS and HRGC-PBMS. The low resolution of

HRGC for these type of high-boiling triterpenes is insufficient for unequivocal HRGC-MS structural assignments. HPLC is selective enough for separation but structural information may only be gained if full mass spectra are obtained. With appropriate tuning, the particle beam HPLC-MS interface is invaluable in obtaining quality mass spectral data and a powerful complementary technique for analysis and structural elucidation of natural products in complex matrices.

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Ms received: March 1, 1996;
accepted: May 30, 1997