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SEPARATION AND CHARACTERIZATION OF OLEANENE-TYPE PENTACYCLIC TRITERPENES FROM *GYPSOPHILA ARROSTII* BY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Saponins were extracted from the roots of *Gypsophila arrostii*. Purified aglycones recovered from acid hydrolysis of the saponins were separated by reversed-phase high-performance liquid chromatography. Three major aglycone components were observed by diode-array detection of UV absorption at 190–200 nm. Further analysis by direct liquid-inlet liquid chromatography–mass spectrometry indicated that the components were triterpenes with molecular weights of 486, 486 and 470. The fragmentation patterns were similar to chemical ionization mass spectra obtained with methane as the ionizing medium. By off-line electron impact mass spectra supported by ^{13}C NMR data, the least polar component (m/z = 470) was identified as gypsogenin. The two other components were tentatively identified as hydroxylated gypsogenin isomers.

INTRODUCTION

Saponins are a group of natural products consisting of a triterpenoid or a steroid aglycone connected to one or two polysaccharides through an ester or a glycosidic linkage. The glycosides, frequently occurring in complex mixtures, are widely distributed in the plant kingdom. Saponins are of interest because of their diverse biochemical activity. Their modifying effect on cell membranes is well known, and the strong haemolytic activity is one of the characteristic properties of saponins[1–3]. Saponins have a potential as pharmaceutical synthons and have been used in hormone synthesis[4].

The background to this work is a study on the characterization of aglycones from *Gypsophila arrostii* (family Caryophyllaceae) which was initiated and supervised by Dr. Jan Karlsen at the Institute of Pharmacy, University of Oslo[5]. The present paper describes the separation and characterization of pentacyclic triterpenoid aglycones from *Gypsophila arrostii* by on-line liquid chromatography–mass spectrometry

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(LC–MS) after hydrolysis of the extracted saponins. A number of separations of saponins or their corresponding aglycones have been reported, using paper chromatography (PC), thin-layer chromatography (TLC)[6–10]. gas chromatography (GC, GC–MS)[11–13] and droplet counter-current chromatography (DCCC)[14–18]. Mass spectrometry (MS)[12,13,19–24] and nuclear magnetic resonance (NMR) spectroscopy[23–25] are important tools for identification of the aglycones. Some reports exist on the high-performance liquid chromatographic (HPLC) separation of pentacyclic triterpene aglycones[26–28], but in none of these the mass spectrometer was used, as an on-line detector. On-line LC–MS offers a short analysis time, and gives structural information on each component without preceding derivatization.

EXPERIMENTAL

Extraction and hydrolysis

Dried roots of *Gypsophila arrostii* (500 g) were extracted with methanol in a Soxhlet apparatus for 72 h. The material (20 g) which precipitated from the methanol solution was dissolved in water and extracted four times with *n*-butanol. The aqueous phase was dried under vacuum, dissolved in a minimum volume of methanol and purified by slow precipitation at 5°C. The last step was repeated once, resulting in 5 g of solid material. A sample of the precipitate was separated by TLC [silica, *n*-butanol–ethanol–water (7:2:5)] and showed four components after detection with antimony trichloride–chloroform. The latter reagent reacts selectively with terpenoid compounds[29], yielding a purple colour with most triterpenes. The two major components were found to have strong haemolytic activity upon coating the developed TLC plates with agar containing 10% (v/v) horse blood and detecting the decolourized zones.

The precipitate was hydrolyzed in methanol–2 *M* aqueous hydrochloric acid (1:1) for 6 h at 20°C. The water-insoluble compounds like aglycones (pentacyclic triterpenes) were precipitated during hydrolysis and recovered by filtration. The carbohydrates were subsequently recovered from the filtrate. Details of the extraction, hydrolysis and TLC separation procedures can be found elsewhere[5].

Instrumentation

The HPLC equipment consisted of a solvent-delivery system (Waters Model 590), a valve loop injector (SSI Model 235) connected to a C_{18} column (Brownlee MPLC™: 100 mm × 2.1 mm, 5-μm RP-18 silica) and a diode-array UV detector (Hewlett-Packard 1040A). Water (deionized and distilled), acetonitrile (HPLC grade S, Rathburn Chemicals) and formic acid (Merck p.a.) were used as the mobile phase.

The spectrophotometer was connected to a double-focusing mass spectrometer (JEOL JMS-DX 303) through a 0.5-μm line filter and a direct liquid-inlet (DLI) interface. In order to isolate the 3 kV acceleration voltage of the ion source from the ground potential with minimum extra-column band broadening, the line filter and the DLI were interconnected through a 30 cm × 50 μm I.D. fused-silica capillary. The DLI, which is described in detail elsewhere[30], was equipped with a 12-μm glass orifice. A stable jet was obtained with flow-rates of 100–200 μl/min. Of the mobile phase 10–50% was split to waste after continuously flushing the rear side of the orifice. Measurement conditions for the mass spectrometer: acceleration voltage, 3

kV: ionizing voltage, 200 eV; ionizing current, 300 μA and electron multiplier voltage, 1.5 kV. Data were processed by the JMA 5000 data system.

RESULTS AND DISCUSSION

NMR and MS characterization of the aglycone mixture

Prior to further analysis by HPLC, the purified water-insoluble mixture, containing the free sapogenins recovered from the hydrolysis, was subjected to direct characterization by NMR and MS. The ^{13}C NMR spectrum of the free acids was in good agreement with chemical shift values previously reported for gypsogenin methyl ester having an olean-12-ene skeleton[25], with deviations from the reported values within 0.8 ppm for 25 of 29 carbon atoms (standard deviation = 0.3 ppm), the ester carbon excepted. The remaining four peaks (one methyl- and three bridgehead carbons) could not be discerned from the background. Functional groups like aldehyde (δ = 207.3 ppm), carboxylic acid (δ = 180.4 ppm), carbon double bond (δ = 122.1, 144.0 ppm) and alcohol (δ = 71.8 ppm) were clearly identified.

A typical feature in electron impact (EI) mass spectra of Δ^{12}-unsaturated pentacyclic triterpenes is the retro-Diels-Alder (rDA) fragmentation illustrated in Fig. 1[21,23,24]. Moving the double bond to other positions will drastically alter the general fragmentation, whereas a hydroxyl group at C-3, substituents at C-17 or an additional double bond in ring D or E (Fig. 1) can lead to changes in the relative intensities, but not in the overall pattern[31]. It has been demonstrated that the rDA fragment *a* of Δ^{12}-oleanene derivatives will usually have a nominal mass of 203 plus the mass of the functional groups belonging to ring C, D or E[20]. The rDA fragments can therefore be a useful tool in the determination of substituent positions.

EI-MS of the hydrolyzed mixture showed prominent fragment ions at *m/z* 248,

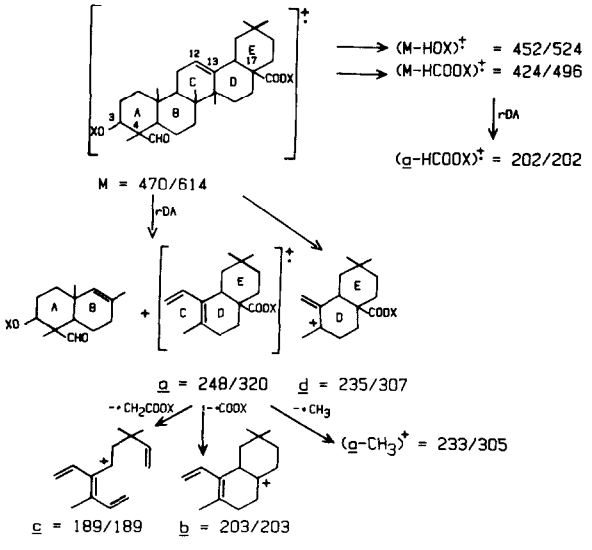

Fig. 1. EI–MS fragmentation of gypsogenin. Two *m/z* values are given for each fragment: underivatized gypsogenin (X = H)/trimethylsilyl-derivatized gypsogenin (X = TMS).

203 and 189 (relative abundances 99, 100 and 40% respectively) consistent with rDA fragments *a*, *b* and *c* in Fig. 1 when X = H. Furthermore, molecular ions with *m/z* 470 and 486 were found, the former indicating gypsogenin and the latter gypsogenic acid and/or hydroxylated gypsogenin isomer.

The identification of seven monosaccharides (rhamnose, fucose, xylose, arabinose, glucose, galactose and glucoronic acid) has been reported for gypsoside[32–34]. The same monosaccharides were identified in the aqueous solution after hydrolysis by TLC separation on cellulose [*n*-butanol–acetic acid–diethyl ether–water (9:6:3:1)], detection with aniline phthalate and comparison with standard monosaccharides.

HPLC separation

The aglycone mixture obtained by acid hydrolysis of saponins was separated on a C_{18} column eluted with acetonitrile–aqueous formic acid. Chromatographic and UV spectroscopic data obtained from the diode-array detector are illustrated in Fig. 2. Four major components could be distinguished at 190 nm (peaks 0, 1, 2 and 3). Of note in the three dimensional isoabsorbance plot is the complete absence of conjugated chromophores in the aglycone sample. Components 2 and 3 had almost identical UV absorption properties, reaching $\lambda_{max} \leqslant 190$ nm, while λ_{max} was found at 196 nm for component 1. The UV scans were as expected for oleanene type compounds containing an isolated double bond. Component 0, readily distinguishable by its low absorptivity at 200 nm (Fig. 2), could be removed from the sample mixture by partitioning the dry sample in ethyl acetate–water (1:1) or extracting an acetonitrile–water (4:6) sample solution with an equal volume of ethyl acetate. Components 1, 2 and 3 could be reconstituted from the organic phase with recoveries higher than 99%. The identity of component 0 was not confirmed, but its high polarity and UV absorption indicate residual carbohydrates from the saponin hydro-

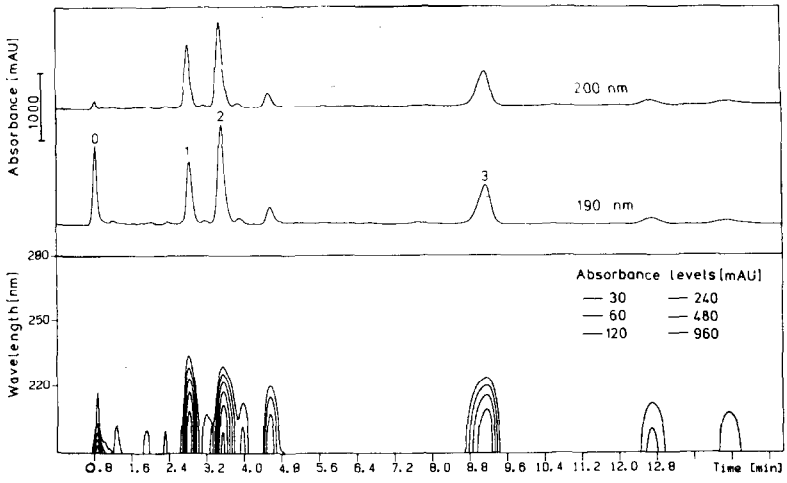

Fig. 2. Reversed-phase HPLC separation of an aglycone mixture obtained by hydrolysis of saponins from *Gypsophila arrostii*. Upper half: Single-channel chromatograms; UV detection at 190 and 200 nm. Lower half: three-dimensional isoabsorbance diagram; UV diode-array detection. Sample size: 1 μl/1 μg. Column: C_{18} (Brownlee, 5-μm RP-18 silica, 100 mm × 2.1 mm I.D.). Mobile phase: acetonitrile–4 m*M* aqueous formic acid (6:4); 0.2 ml/min.

lysis. Further support for this is given by the fact that prolonged exposure of component 0 to hydrolysis did not give rise to observable amounts of aglycone compounds.

The UV absorption ratio scans illustrated in Fig. 3 indicate high spectroscopic homogeneity of components 1 and 3, whereas an impurity is observed on the rear slope of peak 2.

LC–MS

To obtain structural information on the components separated by HPLC, the sample was analyzed by LC–MS, using the acetonitrile–water–formic acid mobile phase as chemical ionization (CI) reagent medium. The total ion current (TIC) chromatogram of the aglycone sample after removal of component 0 is illustrated in Fig. 4.

Components 1 and 2, which appear completely separated by UV detection (Fig. 2), seem not to be resolved as monitored by TIC. This may be an artifact created by the mass spectrometer, as can be seen from the ion chromatogram of the mass at *m/z* 487 (Fig. 4) which displays two baseline-separated peaks; 487 is the nominal mass of the protonated molecular ion (M + H) of components 1 and 2. The strong tailing observed in the TIC is most probably caused by thermal sample degradation

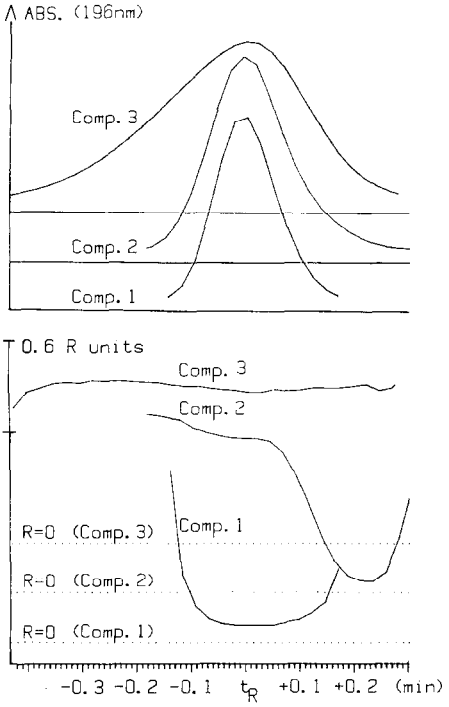

Fig. 3. UV spectroscopic homogeneity of aglycone components. Upper half: chromatographic peak profiles of separated aglycone components; UV detection at 196 nm. Lower half: UV absorption ratio of aglycone components, $R = A$ (194 nm)/A(200 nm). Component numbers refer to peak numbers in Fig. 2. t_R = retention time.

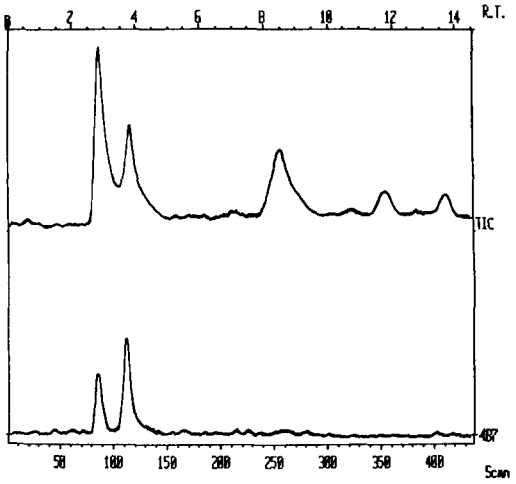

Fig. 4. Direct liquid-inlet LC–MS of an aglycone mixture. Upper trace: total ion current (TIC) chromatogram. Lower trace: ion chromatogram; m/z 487. R.T. = Retention time (min). Sample size: 1 μl/1 μg. LC conditions as in Fig. 2; 30% mobile phase split to waste. MS conditions: ion source temp.: 250°C. Desolvation chamber temp.: 300°C. Other parameters as given in Experimental section.

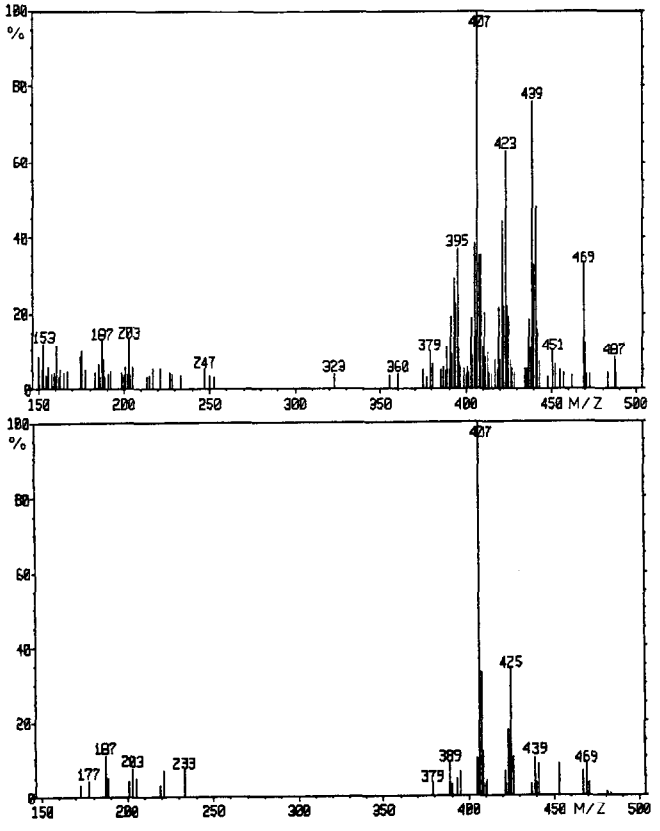

Fig. 5. CI mass spectra of component 1. Upper: on-line LC–MS acetonitrile–4 mM aqueous formic acid (6:4). Lower: off-line CI (methane). Component numbers refer to peak numbers in Fig. 2.

on the hot surface of the desolvation chamber (300°C) and/or the ion source (250°C), giving rise to a delayed emission of ions in the lower m/z range. This observation emphasizes the need for better phase-transition techniques in LC–MS.

On-line LC mass spectra as well as off-line CI (methane) spectra of components 1, 2 and 3 are shown in Fig. 5, 6 and 7 respectively. The fragment ions are identified in Tables I and II. The CI spectra of component 3 are in agreement with the structure of gypsogenin (Fig. 1). The main fragmentation mechanisms are loss of polar substituents as neutral molecules (H_2O, HCOH, CO_2, HCOOH) (Table II). The observed decarboxylation may be a thermal reaction, as changes in relative intensities of the corresponding ions were observed with changing temperature. This was most pronounced in the off-line spectra of the most polar components (1 and 2), as temperatures in excess of 300°C had to be applied to the probe to get a reasonable evaporation rate.

Components 1 and 2 were found to yield similar CI spectra with a protonated molecular ion (M + H) 16 mass units higher than that of component 3, indicating an oxidized aldehyde function (gypsogenic acid) or an hydroxylated gypsogenin isomer. The latter is confirmed by fragment ions of major abundance formed by loss of formaldehyde and one or two water molecules (m/z 439, 421), and the base peak (m/z

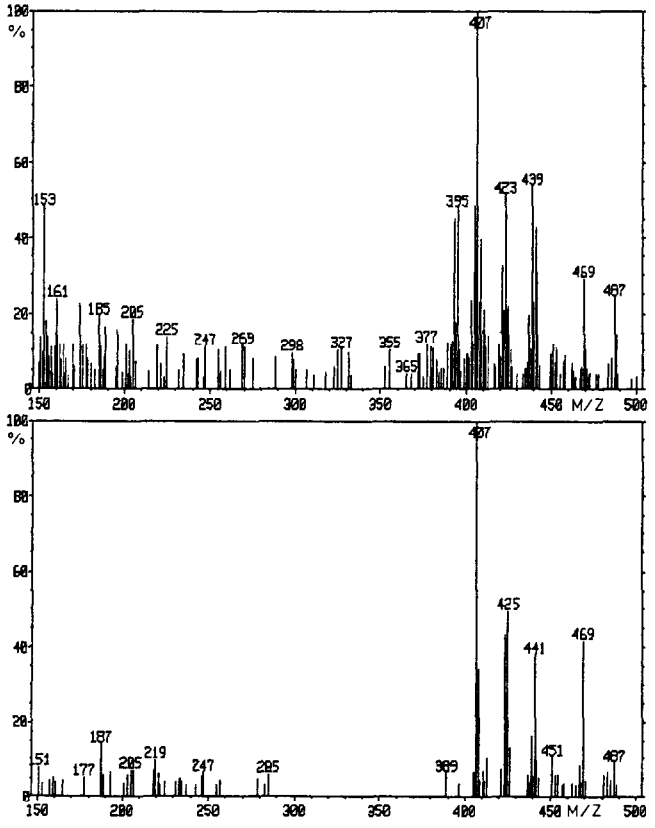

Fig. 6. CI mass spectra of component 2. Details as in Fig. 5.

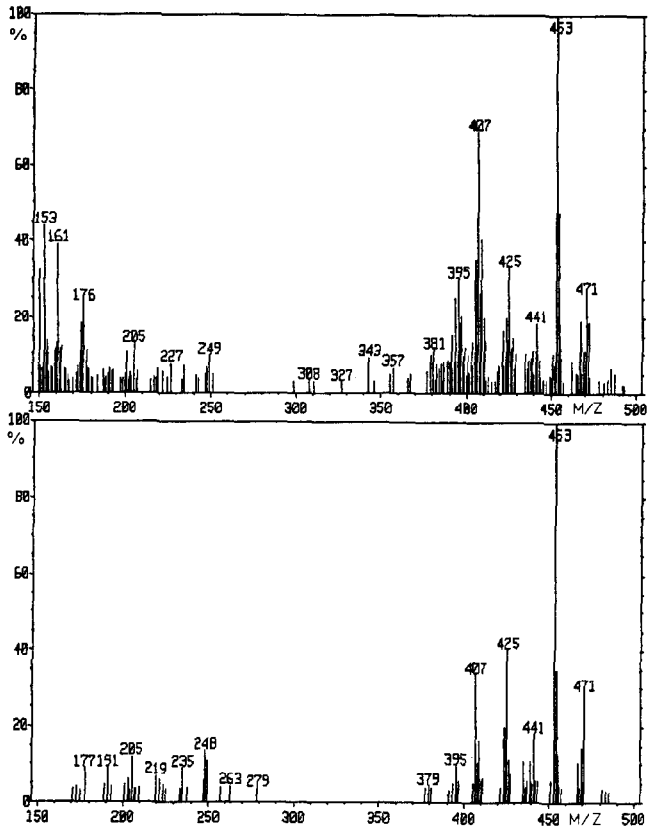

Fig. 7. CI mass spectra of component 3. Details as in Fig. 5.

TABLE I

IDENTIFICATION OF FRAGMENT IONS IN CI MASS SPECTRA OF COMPONENTS 1 AND 2

$[M+H] = m/z$ 487.

Ion *m/z*	*Neutral molecules lost (m/z)*			
	H_2O (18)	*HCOH (30)*	*CO_2 (44)*	*HCOOH (46)*
469	1			
451	2			
441				1
439	1	1		
425	1		1	
423	1			1
421	2	1		
407	2		1	
405	2			1
395	1	1	1	
393	1	1		1

TABLE II

IDENTIFICATION OF FRAGMENT IONS IN CI MASS SPECTRA OF COMPONENT 3

[M + H] = *m/z* 471.

Ion m/z	*Neutral molecules lost (m/z)*			
	H_2O (18)	*HCOH (30)*	*CO_2 (44)*	*HCOOH (46)*
453	1			
441		1		
425				1
423	1	1		
409	1		1	
407	1			1
397		1	1	
395		1		1

407) which corresponds to the oleanene skeleton with one remaining aldehyde group (Table I).

In Fig. 8 are illustrated the selected-ion monitoring (SIM) chromatograms of *m/z* values corresponding to the assumed (M + H), (M + H − H_2O) and gypsogenin skeleton (*m/z* 407) ions. Also shown is *m/z* 483 which might be the (M + H − H_2O) ion of hydroxylated gypsogenin methyl ester. Methyl esters could be formed during acid hydrolysis of saponins in the presence of methanol. No further identification was attempted for these components due to low sample concentrations.

Off-line EI–MS

Components 1 and 2. The EI spectrum of underivatized component 2 given in

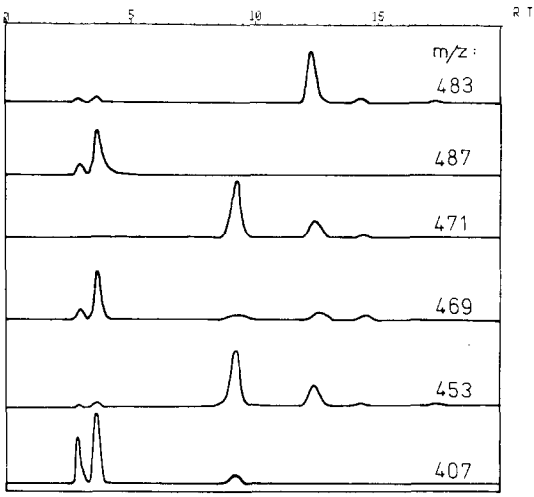

Fig. 8. LC–MS selected ion monitoring (SIM) chromatograms of an aglycone mixture. Conditions as in Fig. 4. Mass microsweep window: 0.25 a.u.; electrostatic field switching. R.T. = Retention time (min).

TABLE III

IDENTIFICATION OF FRAGMENT IONS IN THE EI MASS SPECTRA OF UNDERIVATIZED AND TMS-DERIVATIZED COMPONENT 2

Fragment abbreviations refer to Fig. 1 with an hydroxyl group added to ring C, D or E.

Ion m/z		*Rel. abundance (%)*		*Identification*
X=H	*X=TMS*	*X=H*	*X=TMS*	
486	702	1	6	M
471	687	–	3	M−CH_3
468	612	1	3	M−HOX
440	584	4	8	M−HCOOX
264	408	5	2	a
246	318	18	45	a−HOX
218	290	47	4	a−HCOOX
203	275	50	12	a−(HCOOX+CH_3)
201	201	38	100	a−(HOX+COOX)
200	200	13	40	a−(HOX+HCOOX)
187	187	70	43	a−(HOX+CH_2COOX)

Table III contains four possible primary rDA fragments which could be formed from hydroxy-gypsogenin with a hydroxyl group at ring C, D or E. The observed ions with *m/z* 264, 246 and 218 could be created by direct rDA fragmentation of this molecule or by elimination of H_2O or HCOOH prior to the rDA reaction. The molecular mass (*m/z* 486) as well as the presence of three trimethylsilyl (TMS)-derivatizable functional groups were confirmed by the TMS spectrum which showed a molecular ion at *m/z* 702 for components 1 and 2. Quillaic acid is a possible structure for component 2, since *m/z* values of 486, 468, 264, 246 and 201 (no intensities given) have been observed in the EI mass spectrum of this compound[12]. The EI mass spectrum of underivatized component 1 was however less conclusive, showing a prominent ion at *m/z* 203 (relative abundance = 40%) with no accompanying rDA fragments at higher mass, indicating a possible structure with all polar substituents at rings A and B. The spectra were not checked for metastable ions.

To locate the polar substituents precisely, further investigations are required. The analytical reversed-phase separation of components 1 and 2 was not readily reproduced on a preparative scale, but further improvements are being made.

Component 3. The expected fragmentation mechanisms of gypsogenin shown in Fig. 1 were established by application of the general principles outlined elsewhere[21–23]. The EI mass spectra of TMS-derivatized and underivatized component 3 given in Table IV are in accord with the structure of gypsogenin. Elimination of neutral molecules from the radical molecular ion is the dominating primary fragmentation, either by polar substituents[19] or through the rDA reaction. The two most abundant ions (fragments *a* and *b*) arise from the rDA reaction. As previously explained, the two peaks with nominal masses 203 and 248 are consistent with the carboxyl function of gypsogenin. It should be noted that rDA fragmentation can also take place after elimination of neutral molecules. This reaction, which is much more feasible with TMS-derivatized component 3 due to enhanced elimination of

TABLE IV

IDENTIFICATION OF FRAGMENT IONS IN THE EI MASS SPECTRA OF UNDERIVATIZED AND TMS-DERIVATIZED COMPONENT 3

Fragment abbreviations refer to Fig. 1.

Ion m/z		*Rel. abundance (%)*		*Identification*
X=H	*X=TMS*	*X=H*	*X=TMS*	
470	614	2	12	M
455	599	1	8	$M-CH_3$
452	524	1	1	M–HOX
424	496	5	20	M–HCOOX
248	320	90	35	a
235	307	2	15	d
233	305	10	–	$a-CH_3$
203	203	100	100	b
202	202	10	60	a–HCOOX
189	189	20	40	c

formic acid TMS ester compared to underivatized formic acid, leads to *m/z* 202. Comparison of the spectra of the underivatized and TMS-derivatized compounds confirms the molecular weight (*m/z* 470), the presence of two groups available for TMS ester/ether formation as well as the nominal masses of the proposed fragments in Fig. 1.

CONCLUSIONS

This work has demonstrated that on-line LC–MS is a useful method for the analysis of pentacyclic triterpenes from biological samples. Although the non-compatibility of current DLI flow-rates with EI ionization remains a drawback, the CI spectra provided information on molecular weights as well as the nature of the polar substituents of three separated aglycones. One aglycone was subsequently identified as gypsogenin by off-line MS techniques. Coupled with diode-array UV data, the LC mass spectra can provide a suitable basis for the selection of components to be subjected to other mass spectrometric or spectroscopic techniques.

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