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Determination of naphthodianthrones in plant extracts from *Hypericum perforatum L.* by liquid chromatography–electrospray mass spectrometry

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Abstract

The determination of the naphthodianthrone constituents in extracts of dried blossoms of *Hypericum perforatum L.* by combined HPLC–electrospray mass spectrometry is described. Hypericin (1), pseudohypericin (2) and their precursor compounds produce intensive negative quasi-molecular ions by deprotonation provided a non-acidic eluent system is used in the HPLC separation. From the $[M-H]^-$ ions formed in the electrospray ionization process characteristic daughter ion spectra can be obtained by collisional activation which have been studied by tandem mass spectrometry. © 1997 Elsevier Science B.V.

Keywords: *Hypericum perforatum*; Naphthodianthrones; Hypericin; Pseudohypericin; Protohypericin; Protopseudohypericin

1. Introduction

Hypericum preparations have been used as medicine since antiquity. In folk medicine St. John's wort is used both for the external treatment of wounds (St. John's wort oil) as well as internally to treat minor depression and mood swings. In various studies the isolation and analysis of a very high number of substances from different structural types and their pharmacological properties have been described [1–3]. In addition to substances like flavonoids, tannic acids, xanthones and phloroglucin derivatives, the naphthodianthrones are particularly important (Fig. 1). Only recently the strong antiviral effect of the ingredients hypericin and pseudohypericin has been discovered [4]. Further studies have shown that, in the presence of light, hypericin and pseudohypericin act as photosensitizers and are thereby capable of destroying surface structures of viruses [5–8].

Very little mass spectrometric data have been published to characterize hypericin and related naphthodianthrones. Occasionally, the detection of $M^{+\cdot}$ ions under field desorption ionization has been reported [9–11]. No electron impact mass spectra are mentioned in the literature. This lack of data is partly due to the low vapor pressure of these substances which calls for evaporation temperatures at the upper tolerance limit of conventional electron impact ion

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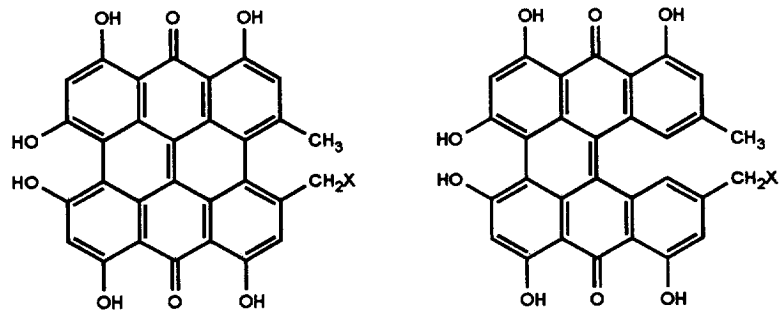

X = H, hypericin (1, M_r = 504)
X = OH, pseudohypericin (2, M_r = 520)
X = H, protohypericin (3, M_r = 506)
X = OH, protopseudohypericin (4, M_r = 522)

Fig. 1. Chemical structure of the naphthodianthrone constituents of *Hypericum perforatum* L.

sources. In our own attempt a 70 eV electron impact mass spectrum of hypericin could be recorded at temperatures above 250°C. It exhibits a $M^{+\cdot}$ peak at *m/z* 504 and the doubly charged molecular ion at *m/z* 252 but no characteristic fragment ions.

Hypericum preparations are usually validated by HPLC determination of hypericin and pseudohypericin as the main constituents. In most published procedures an eluent system with an acidic phosphate buffer was used [12–15]. In some cases neutral eluent systems have been reported as mobile phase [16,17]. The best separation results could be achieved by gradient systems consisting of methanol–acetonitrile-buffer or methanol–ethyl acetate-buffer mixtures. In general, reversed-phase (RP 18) material of medium (4–5 μm) grain size has been recommended as stationary phase.

In this communication we report the characteristic mass spectrometric fragmentation patterns of the naphthodianthrones in *Hypericum* extracts obtained by collision induced dissociation of the corresponding $[M-H]^-$ anions produced by the electrospray technique. By applying suitable selected eluents the mass spectral data may be collected in combined HPLC–tandem MS experiments.

2. Experimental

2.1. Materials

The blossoms of *Hypericum perforatum L.* were collected during the flowering time in July 1995 in Buettelborn, Germany. Water was purified by a Milli Q plus system from Millipore (Milford, MA, USA). Authentic hypericin was obtained from Extrasynthese (Genay, France). Acetonitrile, methanol, triethylamine and acetic acid were purchased from Merck (Darmstadt, Germany). All solvents were gradient grade. The 0.1 *M* triethylammonium acetate buffer was prepared by dissolving 10.1 g triethylamine and 6 g acetic acid in 1 l water. The pH was adjusted to 7 with triethylamine.

2.2. Chromatography

The HPLC system consisted of a ternary pump SP 8800 (Spectra-Physics, Darmstadt, Germany), a Dilutor 401 equipped with a 5 ml syringe and a sample injector M 231 (Gilson Abimed, Langenfeld, Germany). The detection was performed with a multiwavelength spectrometer with a step width of 5 nm and a spectral range of 370–790 nm (Spectra-Physics). The chromatographic data were recorded and processed by the Spectra-Focus software (Spectra Physics). The solvents were degassed by a vacuum degasser SCM 400 (Spectra-Physics). A LiChrosorb RP 18 column (125×4 mm I.D., 5 μm, Merck, Darmstadt, Germany), protected with a LiChrospher 100 RP 18 (4×4 mm I.D., 5 μm, Merck) was used for chromatographic separation. The solvent system consisted of methanol–acetonitrile (5:4) (solvent A) and 0.1 *M* aqueous triethylammonium acetate (solvent B). The starting gradient was composed of 70% A and 30% B. Then the portion of A linearly increased with a flow-rate of 0.6 ml/min to

90% (8 min). The equilibration of the column was then achieved by a linear change to the starting conditions over the next 5 min so that the total analysis time was 13 min. Detection was in the visible range at 590 nm and the injection volume was 20 μl [total concentration of hypericins (1–4) ca. 0.63 mg/ml].

2.3. Mass spectrometry

The electrospray mass spectra were recorded with a Perkin-Elmer Sciex (Norwalk, CT, USA) API III+ triple quadrupole mass spectrometer, equipped with a pneumatically assisted electrospray ionization source. Mass analysis was made by using Q1. Daughter ion spectra were recorded using Q3, after selection of the precursor ions in Q1 and collisionally induced dissociation in Q2. Argon was used as a collision gas at a collision gas density of 2.16×10^{14} atoms/cm^2. The HPLC flow of 600 μl/min was split into a capillary flow of 20 μl/min using a needle valve (Series 1300, Hoke, Frankfurt/Main, Germany). The outlet of the needle valve was directly coupled to the electrospray needle using a 30 cm fused-silica capillary (180 μm O.D., 100 μm I.D.). The coaxial nebulizer gas (air) pressure was 1.1 l/min. Curtain gas (nitrogen) flow-rate was 0.6 l/min. The orifice plate was kept at 50°C for analyses. The operating voltage of the electrospray capillary was −5.0 kV. The interface voltage was set at −650 V, the orifice at −100 V. Other operating voltages were as follows: R0=−40 V, R1=−27 V, R2=0 V, R3=5 V. Resolution parameters were adjusted to achieve unit mass resolution in the mass range 50–600 amu. Mass spectra were recorded in the negative mode (step size 0.5 amu, dwell time 0.5 ms) over the mass range m/z 50–600 amu and acquired to disk. Q1 and Q3 were calibrated in the positive mode using a CsI solution. Data were acquired and processed using the Sciex Mac Spec data system.

The negative CAD mass spectra given in Fig. 3 were recorded by injecting 20 μl of a methanolic extract containing 4.2 μg of hypericin and 12.6 μg of total hypericins, respectively. Due to the splitting ratio applied, only ca. 3% of the material was used for ion production. Mass spectra with still acceptable signal-to-noise ratio could be detected at quantities of 0.1 μg injected hypericins. The reproducibility of the mass spectra was within ±5%.

2.4. Extraction procedure

Air dried blossoms (5 g), stored under darkness were exhaustively extracted under strict exclusion of light with 400 ml of methanol–acetone (1:1) using a Soxhlet extractor until the solvent became colorless. Evaporation of the solvent on a rotary evaporator produced 1.6 g of a dry extract which was dissolved in 50 ml of methanol. For HPLC analysis the solution has to be filtered first.

Using authentic hypericin as reference compound the yield of extracted hypericin was determined by HPLC as 2.1 mg/g dried blossoms. The total quantity of hypericins (1–4) amounted to 6.3 mg/g based on the assumption that the related compounds have the same response factors. The reproducibility of the HPLC runs was within ±6%.

3. Results and discussion

Hypericin and related compounds show very little tendency to form $[M+H]^+$ quasi molecular ions by addition of protons. Therefore, in a series of experi-

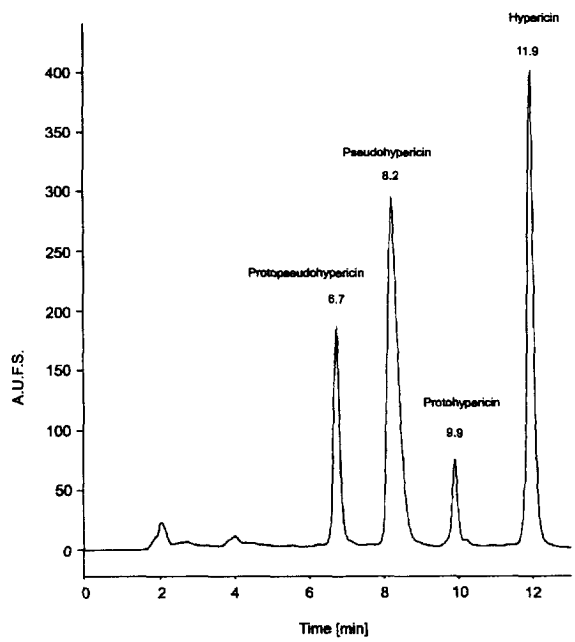

Fig. 2. HPLC chromatogram obtained by injecting 20 μl of a *Hypericum* extract using a methanol–acetonitrile–triethylammonium acetate buffer system (detection at 590 nm).

ments using a wide range of acidic buffers only weak signals of the protonated quasimolecular ions of hypericin could be detected by electrospray ionization. On the other hand, a high yield of $[M-H]^-$ quasi molecular ions is produced by the same technique, however, applying basic buffer systems. Even under neutral conditions the extent of deprotonation is still sufficient to observe high intensities of $[M-H]^-$ ions.

A basic eluent system would be favourable to facilitate the formation of $[M-H]^-$ ions in combined LC–electrospray MS experiments. However, the chromatographic columns used in these experiments tolerate only eluents in the pH range from 2.0–7.5. As mentioned above the deprotonation tendency in neutral solvent systems of the phenolic naphthodianthrones is still sufficiently expressed to produce $[M-H]^-$ ions under electrospray ionization with good signal-to-noise ratio. Furthermore, with acetonitrile–methanol–water–triethylammonium acetate adjusted to pH 7 as eluent, chromatographic separations of the naphthodianthrones in *Hypericum* extracts with a similar quality as observed with the acidic eluents could be achieved (Fig. 2). Thus, mixtures of this kind proved to be systems of choice for combined LC–MS experiments.

To obtain the chromatogram reproduced in Fig. 2 the quantities of the injected hypericin and total hypericins amounted to 4.2 μg and 12.6 μg, respectively. In dilution experiments it was found that the minimum amounts necessary to detect hypericin and the related naphthodianthrones correspond to ca. 0.05 μg. The reproducibility of the determined hypericin was within ±6%.

The negative ion CAD mass spectra of compounds 1–4 reproduced in Fig. 3 were obtained by injecting mixtures containing the individual hypericins in the μg range. In the case of hypericin an unambiguous detection by LC–MS–MS applying multiple ion detection of the quasimolecular ion (*m/z* 503) and the most intense fragment ion (*m/z* 405) could be achieved down to 2 ng of injected hypericin. At quantities ranging from 10 to 1000 ng a linear dependence between the peak areas and the amount of injected hypericin was observed.

The $[M-H]^-$ ions of hypericin and the related

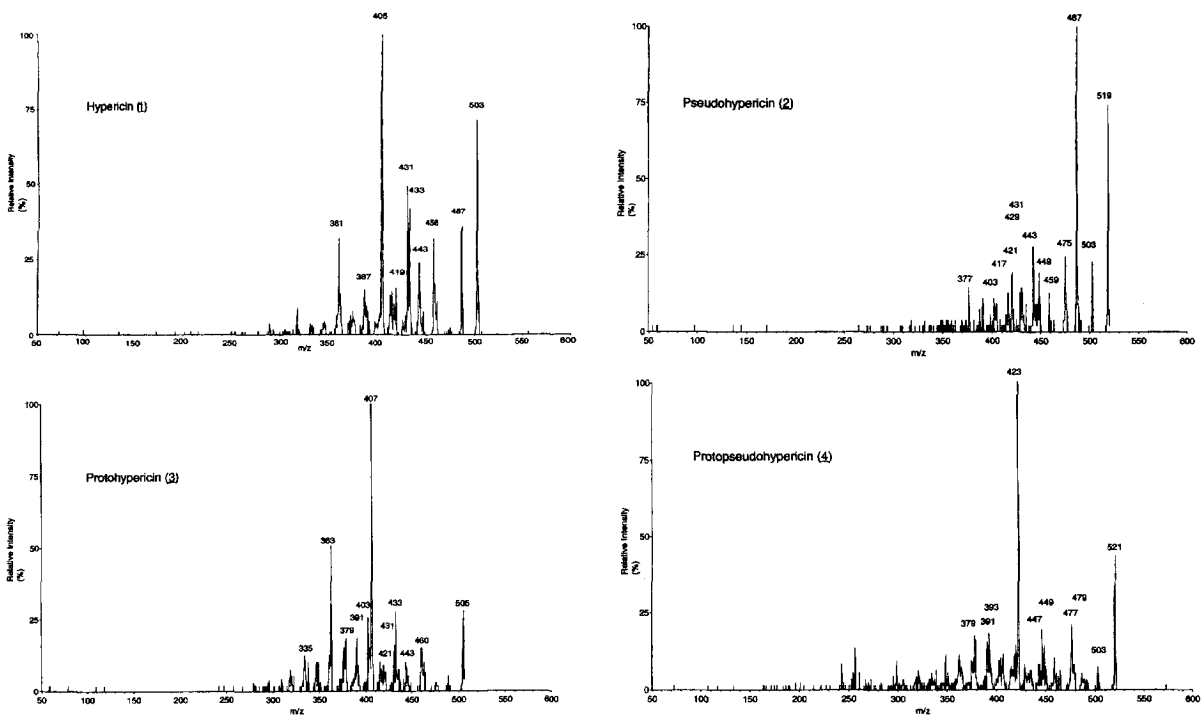

Fig. 3. Negative ion CAD mass spectra of the compounds 1–4 (conditions see Section 2).

substances are very stable closed-shell anions, showing no fragmentation under mild electrospray conditions. Thus, tandem mass spectrometry was used to obtain characteristic fragmentation patterns for this type of compounds.

In the following discussion an interpretation of the collision induced dissociation of the $[M-H]^-$ species of hypericin (1) is presented. The postulated structural representations of the observed fragment ions are partly based on mechanistic studies on simple phenolate model systems [18–20] showing similar structural features as found in the more complex hypericin anion. The fragmentations of the negative quasimolecular ions of the related compounds 2–4 show close similarities and are summarized in Table 1.

The mass differences observed between the quasimolecular anions of hypericin and the daughter ions produced by collision induced dissociation cannot be rationalized assuming a single fragmentation step. However, the stepwise expulsion of two and more stable neutrals during the residence time in the collision chamber of the triple stage mass spectrometer provides a plausible explanation of the measured MS–MS spectra. The high collision gas density applied in the tandem mass spectrometry experiments leads to multiple collisions within the gas cell, thus supplying the necessary energy for fragmentation cascades.

In the literature relatively little is known on the collision induced fragmentation of phenolate anions [18–20]. In the case of resorcinol, the fragmentation of the $[M-H]^-$ ion is partly explained by rearrangement to a tautomeric species [19]. To rationalize the formation of some of the main fragments, a similar tautomerism for the deprotonated hypericin *a* may be assumed (Fig. 4).

The rearranged anion *b* should be able to expel ketene after collisional activation. Although no $[M-H-CH_2=C=O]^-$ is observed, ions corresponding to loss of ketene and CO and 2 CO, respectively give rise to prominent fragment peaks at *m/z* 433 and 405. Further dehydration of the ion at *m/z* 405 could

Table 1
Negative quasimolecular and collision induced fragment ions of compounds 1–4

m/z	Rel. Int. %	Ion	*m/z*	Rel. Int. %	Ion
Hypericin			*Protohypericin*		
503	72	$[M-H]^-$	505	28	$[M-H]^-$
487	36	$[M-H-CH_4]^-$	460	15	$[M-H-CO_2-H\cdot]^-$
458	32	$[M-H-CO_2-H\cdot]^-$	443	10	$[M-H-CO_2-H_2O]^-$
443	24	$[M-H-CH_2=C=O-H_2O]^-$	433	28	$[M-H-CO_2-CO]^-$
433	42	$[M-H-CH_2=C=O-CO]^-$	431	17	$[M-H-2CO-H_2O]^-$
431	50	$[M-H-CO_2-CO]^-$	421	9	$[M-H-2CH_2=C=O]^-$
419	16	$[M-H-2CH_2=C=O]^-$	407	100	$[M-H-CH_2=C=O-2CO]^-$
405	100	$[M-H-CH_2=C=O-2CO]^-$	403	26	$[M-H-3CO-H_2O]^-$
387	15	$[M-H-CH_2=C=O-2CO-H_2O]^-$	379	19	$[M-H-CH_2=C=O-3CO]^-$
361	32	$[M-H-CH_2=C=O-CO_2-2CO]^-$	363	51	$[M-H-CH_2=C=O-CO_2-2CO]^-$
Pseudohypericin			*Protopseudohypericin*		
519	70	$[M-H]^-$	521	44	$[M-H]^-$
503	19	$[M-H-CH_4]^-$	503	8	$[M-H-H_2O]^-$
487	100	$[M-H-CH_3OH]^-$	479	9	$[M-H-CH_2=C=O]^-$
475	24	$[M-H-CO_2]^-$	477	21	$[M-H-CO_2]^-$
459	12	$[M-H-CH_2=C=O-H_2O]^-$	449	15	$[M-H-CO_2-CO]^-$
449	19	$[M-H-CH_2=C=O-CO]^-$	447	20	$[M-H-2CO-H_2O]^-$
443	28	$[M-H-CH_2=C=O-H_2O-CH_4]^-$	423	100	$[M-H-CH_2=C=O-2CO]^-$
431	14	$[M-H-2CO_2]^-$	391	15	$[M-H-CH_2=C=O-2CO_2]^-$
429	13	$[M-H-CO_2-CO-H_2O]^-$	379	18	$[M-H-CH_2=C=O-CO_2-2CO]^-$
421	19	$[M-H-CH_2=C=O-2CO]^-$			
417	13	$[M-H-3CO-H_2O]^-$			
403	11	$[M-H-CH_2=C=O-2CO-H_2O]^-$			
377	15	$[M-H-CH_2=C=O-CO_2-2CO]^-$			

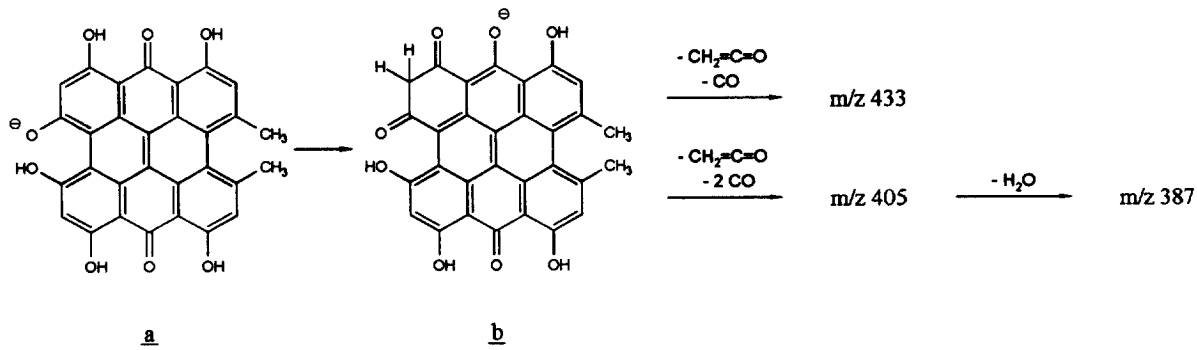

Fig. 4. Collision induced tautomerization of the $[M-H]^-$ ion of hypericin followed by ketene and associated eliminations.

lead to the formation of m/z 387. The eliminated CO molecules could originate either from the carbonyl or the phenolic function [18,20] of hypericin.

Ketene elimination may also be involved in the formation of the ion at 443. Its mass difference to the $[M-H]^-$ ion corresponds to acetic acid, which may best be rationalized by loss of ketene and water. The elimination of water from the anion of resorcinol yielding benzyne-like structures is well documented in the literature [18]. Ion *c* may be used as structural representation of the $[M-H-CH_2=C=O-H_2O]^-$ anion. The ion at m/z 419 could result from elimination of two ketene molecules (Fig. 5).

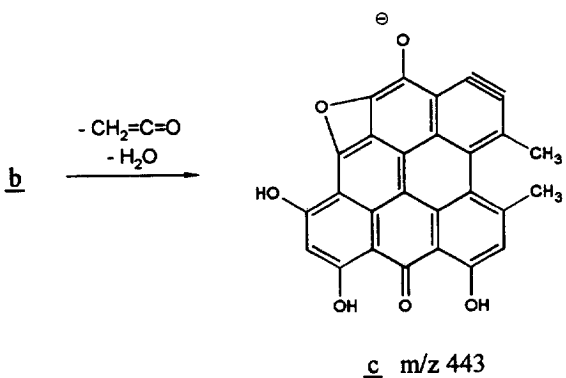

Fig. 5. Ketene and water elimination from the tautomerized $[M-H]^-$ ion of hypericin.

In the literature [19] it has been reported that the $[M-H]^-$ ion of resorcinol can eliminate CO_2. This fragmentation has been explained by a preceding rearrangement to a 6-methylenepyranone anion. We propose a similar process for hypericin to rationalize a further series of fragments (Fig. 6).

Expulsion of CO_2 and in addition CO from the rearranged ion *d* would explain the intense peak at m/z 431.

The occurrence of the fragment at m/z 458 (Fig. 7) with an even mass number indicates the formation of a radical anion from the closed shell $[M-H]^-$ anion at m/z 503. For energetic reasons, a process of

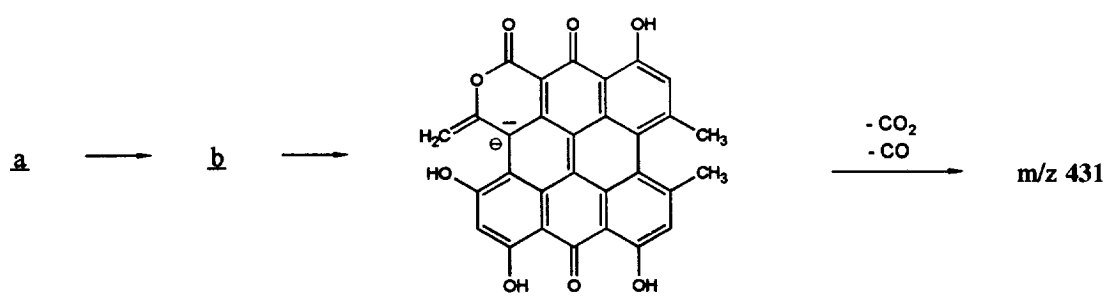

Fig. 6. CO_2 and associated eliminations after rearrangement of the $[M-H]^-$ ion of hypericin.

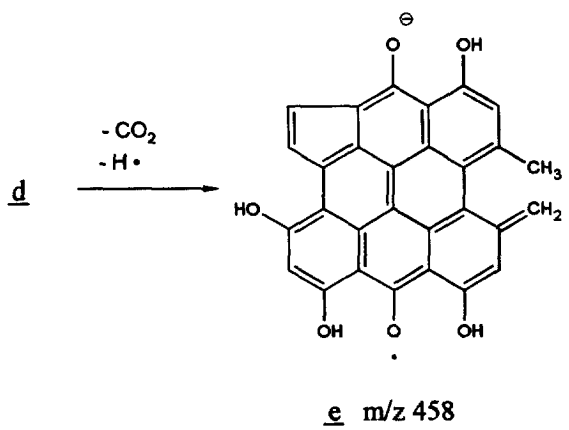

Fig. 7. Formation of the radical-anion at m/z 458.

this kind is only possible when a particularly stable radical anion results. The mass difference corresponds to loss of CO_2H which could be attained by elimination of CO_2 and a hydrogen atom. The production of a semiquinone-like species *e* (or a similar structure) is suggested to account for this fragmentation.

The fragment ion at m/z 487 differs from the $[M-H]^-$ ion by 16 mu. This may be rationalized by elimination of one of the methyl groups as methane (Fig. 8), which is supported by the equivalent loss of 32 mu, corresponding to CH_3OH, from the $[M-H]^-$ ion of (2). The involvement of both sterically neighboured methyl groups in this fragmentation process may yield the ionic structure *f*.

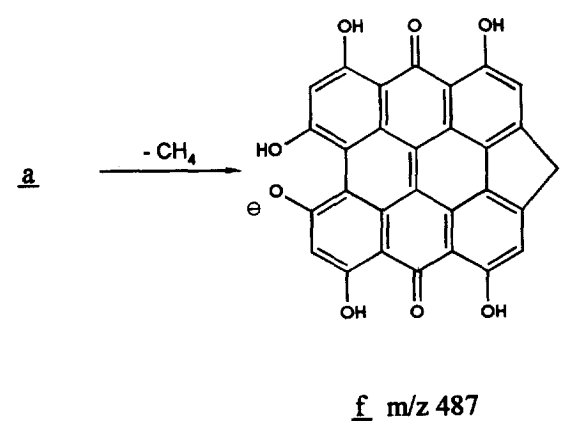

Fig. 8. Methane elimination from the $[M-H]^-$ ion of hypericin.

4. Conclusions

It has been demonstrated that from a single HPLC run (total expertise time: 13 min) characteristic MS–MS data of the naphthodianthrones of *Hypericum* extracts (St. John's wort) extracts can be obtained. Since the determination of hypericin and related compounds are generally used for the characterization and standardization of extracts and phytotherapeutic products of *Hypericum perforatum L.* the described method may be of analytical importance.

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