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Analysis of secreted flavonoids of *Cistus ladanifer L.* by high-performance liquid chromatography–particle beam mass spectrometry

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Abstract

The analysis of secreted flavonoids of the leaf *Cistus ladanifer* was carried out by high-performance liquid chromatography–mass spectrometry coupled through a particle beam interface. The mass spectra were recorded using electron impact. Apigenin, 4′-methyl-apigenin, 7-methyl-apigenin, 7,4′-dimethyl-apigenin, 3-methyl-kaempferol, 3,4-dimethyl-kaempferol, 3,7-dimethyl-kaempferol and 3,7,4′-trimethyl-kaempferol were identified in the leaf resin. © 1998 Elsevier Science B.V.

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1. Introduction

Flavonoids are regarded as one of the most numerous and widespread groups of natural constituents found in plants. Flavonoids have been found to occur practically in all parts of plants. The flavonoid family consists of many subclasses, including flavones, flavanones, isoflavones, chalcones, aurones, anthocyanins, etc. Identification of these subclasses has been recognized as being very useful in chemotaxonomic studies.

The genus *Cistus Ladanifer* is a characteristic genera of the mediterranean region [1]. A previous study on flavonoid glycosides from *Cistus* showed that the flavonoid patterns were rather uniform throughout the whole genus and did not allow a clear distinction between species or groups of related species based on qualitative differences [2]. Several species of *Cistus* secrete large amounts of resin on the surfaces of leaves and stems, and the secreted flavonoids show very distinct patterns that might be useful in delineation of these species [3].

Most plants produce from a few to several dozen flavonoids whose structural similarities make isolation, separation and purification of each substance, in milligram quantities prior to identification, a tedious and difficult task. Thus, a routine analysis method that allows the identification of flavonoids, while avoiding unnecessary and costly isolations, could be a useful tool in chemotaxonomic studies and in studies dealing with their role in plant physiology or in plant biochemistry. With this in mind, high-performance liquid chromatography coupled on-line with mass spectrometry (LC–MS) could be the analytical technique of choice for the identification

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of flavonoids in crude plant extracts, without preliminary isolation and/or chemical treatment.

In recent years, several new LC–MS interfaces have become commercially available, increasing the range of compounds amenable to analyses by LC–MS; one of these being the particle-beam (PB) interface [4]. It has been clear from the start that availability of libraries of electron impact (EI) mass spectra and the characteristic fragmentation of organic molecules in the EI mode made the PB interface very useful for the identification of non-target constituents [5].

The aim of this work was to study the applicability of LC–PB-MS for identification of the secreted flavonoids of *C. ladanifer L.*, without the need for prior purification of each compound.

2. Experimental

2.1. Plant material and sample preparation

A group (30 one-year-old plants) of *C. ladanifer* plants was selected in the country of Alburquerque (Badajoz, Spain). Three samples were taken from each plant, each weighing between 0.25 and 0.30 g (three–four leaves). The selected leaves sprouted in spring, while the samples were collected in August, when maximum secretion of the flavonoids takes place [6].

Sample pretreatment of the exudate was performed by placing the leaves in fresh chloroform (2 ml×5) for 1 min, to ensure the complete extraction of these compounds [7]. To remove the waxes from the resinous material, the chloroform extract was evaporated to dryness and redissolved in methanol (50–60°C) (1 g of extract in 50 ml of methanol). Additionally, the solutions were cooled to −20°C and stored overnight. This resulted in precipitation of the waxes, which were separated from the soluble resin. After evaporation of the methanol, the crude extract containing the flavonoids was directly chromatographed on Sephadex LH-20 (12.5 g Sephadex LH-20; column, 25 cm long, with a diameter of 1.5 cm) with methanol as the eluent at a flow-rate of 1 ml/min. Terpenoids were eluted first and identified by thin-layer chromatography (TLC) on silica gel 60 [7]. The flavonoid fraction was detected at 350 nm with a UV lamp.

2.2. Structural characterization of plant extracts

HPLC analyses were performed using a Beckman 126 programmable solvent module on-line with a UV Beckman 168 diode array detector module (Fullerton, CA, USA). A 250×4.5 mm I.D., 5 μm reversed-phase C_{18} LiChrosorb column was used for separation. Analyses were carried out using 0.1 *M* ammonium acetate in water–tetrahydrofuran–methanol–acetonitrile (56:22:16:6, v/v) as the mobile phase at a flow-rate of 0.8 ml/min.

LC–PB-MS analyses were carried out by fitting the UV detector on-line with a Vestec universal interface (Houston, TX, USA). This interface encompasses a vaporizer with a replaceable tip, a membrane diffusion cell and a dual-stage momentum separator. The diffusion cell was connected to the momentum separator via PTFE tubing (approx. 1 m long, 6 mm O.D. and 4 mm I.D.). The first stage of the momentum separator was pumped with a 25 m^3/h- and the second stage with a 12 m^3/h Edwards direct drive mechanical pump (Crawley, UK). The output of the momentum separator was connected to a standard EI/CI ion source of the VG MS 30/70 double focusing mass spectrometer (VG Analytical, Manchester, UK) via a quartz tube (6 mm O.D., 2 mm I.D.). The temperature of the momentum separator was 150°C and the ion source temperature was maintained at 220°C. An optimal setting of the universal interface was obtained in flow injection mode using a 0.1 μg/μl solution of apigenin, a well-known constituent of *C. ladanifer* [3] with the following settings: Helium carrier gas, 40 ml/min; helium sweep gas, 60 ml/min; tip temperature, 120°C; spray temperature, 80°C; separator temperature, 50°C. The MS conditions were as follows: Ion source temperature, 220°C; electron energy, 70 eV; acceleration voltage, 4 kV; trap current, 100 μA. The MS was set on EI^+ and full scan mode. Mass spectra of pure individual compounds were determined under the same MS conditions by direct solid probe insertion.

3. Results and discussion

Bellar et al. [8] have reported significantly enhanced ion abundances in LC–PB-MS systems if a semi-volatile carrier is added to the mobile phase.

The word ''carrier'' suggests that the component has the effect of increasing analyte transfer efficiency. Initial LC–PB-MS experiments to determine optimum conditions, using ammonium acetate at concentrations varying from 0.01 to 0.1 *M* in the mobile phase, were carried out. At a 0.1 *M* ammonium acetate concentration, using apigenin as a reference standard, the MS sensitivity was increased by a factor of eight. Under the experimental conditions reported, the EI mass spectra of flavonoids from crude extract, obtained by LC–PB-MS, showed fragments which suggested that these ions could result from impurities derived from plant extraction. A chromatography step through Sephadex LH-20 columns, using methanol as the eluent, was used to clean the extract, prior to analyses by LC–PB-MS [7].

A total ion chromatogram of *C. ladanifer* exudate is shown in Fig. 1. In EI-MS, most flavonoid aglycones yield intense peaks for the molecular ion

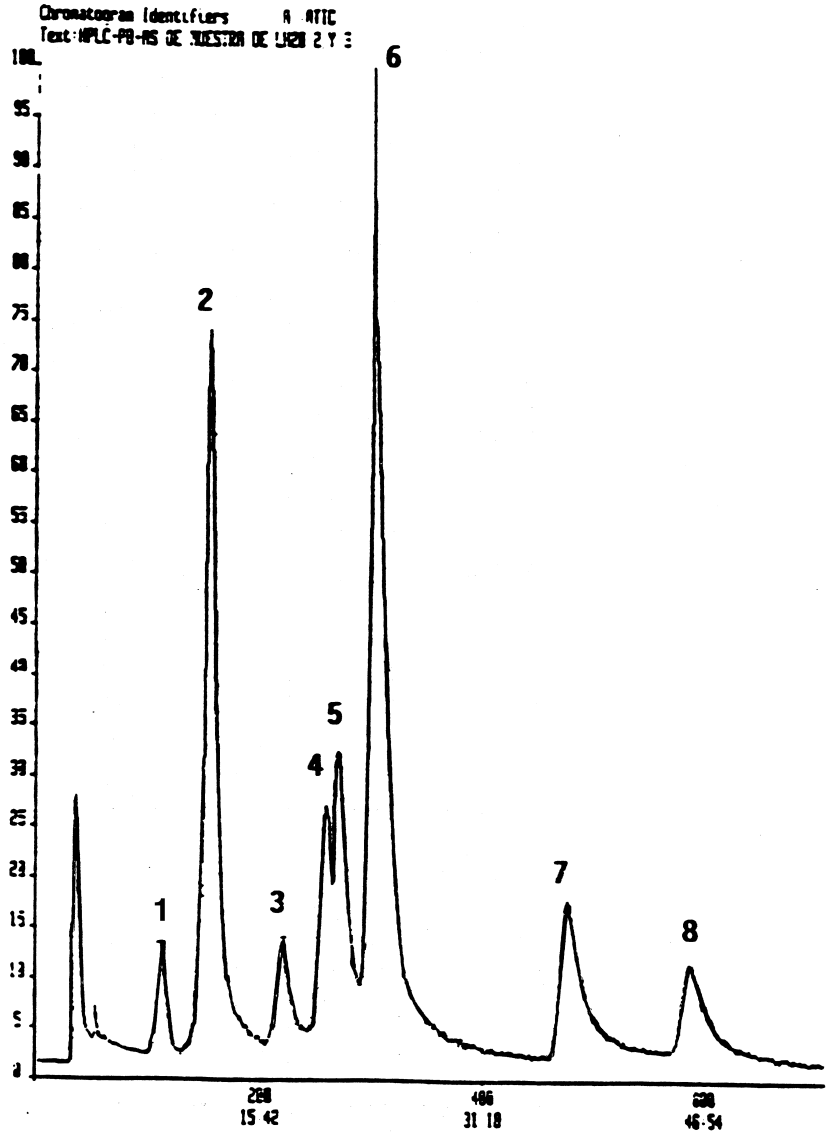

Fig. 1. LC–PB–EI-MS total ion chromatogram of *Cistus ladanifer* flavonoids.

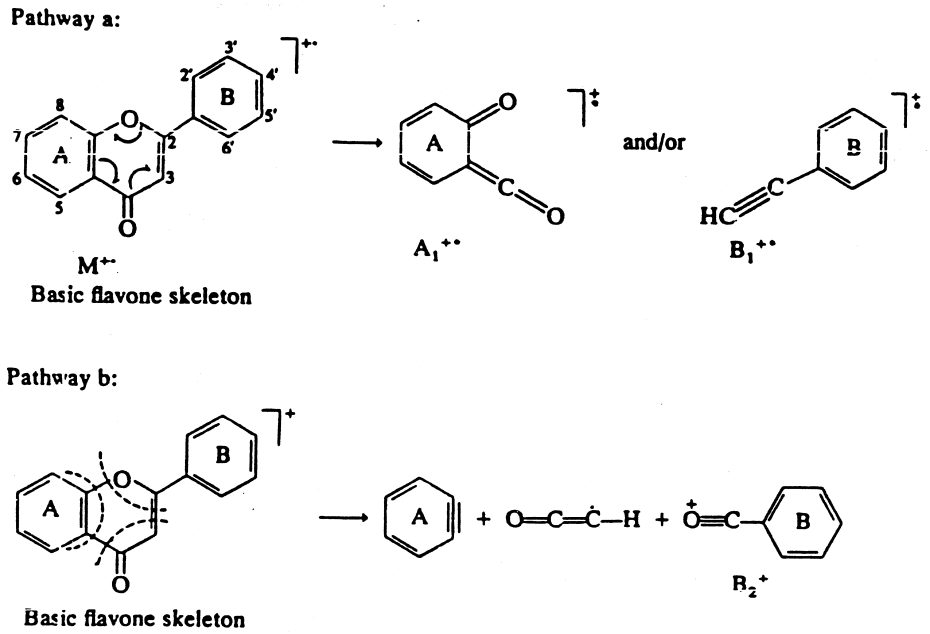

Fig. 2. Diagnostic mass spectral fragmentation pathway of flavonoids.

($M^{+\cdot}$) and, indeed, this is often the base peak. During EI, in addition to the molecular ion, flavonoids are cleaved into many intact A- and B-ring fragments. Two important fragmentation processes of flavonoids (Fig. 2) are designed as pathway a, a retro-Diels-Alder (RDA) cleavage, and pathway b; the fist process produces two RDA ions, $A_1^{+\cdot}$ and $B_1^{+\cdot}$, in contrast, the second one yields the ion B_2^+. The combination of these ions appears to be characteristic for each class of flavonoid [9,10]. The systematic nomenclature developed by Mabry and Markham [9] and Grayer [11] to define and tabulate the ring fragments was used in this study. Table 1 reports the EI diagnostic fragments and their relative intensities for the flavonoids under study. The base peak for all flavonoids is the molecular ion $M^{+\cdot}$. The fragments $A_1^{+\cdot}$, $[A+1]^+$, $B_1^{+\cdot}$ and B_2^+ are present in all spectra. These diagnostic ions only occur in flavones, isoflavones and flavonols [11]. Additionally, Mabry and Markham [9] have found other diagnostic ions in flavonols, including $[M-H]^+$, $[M-H_2O]^+$, $[M-OCH_3]^+$ and $[M-CH_3CO]^+$.

Table 1
LC–PB–EI–MS diagnostic ions in the spectra of the flavonoids investigated, together with their relative intensities (%)

Compound number[a]	$M^{+\cdot}$	$[M-1]^+$	$[M-H_2O]^{+\cdot}$	$[M-CO]^{+\cdot}$	$[M-CHO]^{+\cdot}$	$[M-OCH_3]^+$	$[M-CH_3CO]^+$	$A_1^{+\cdot}$	$[A_1-H]^+$	$B_1^{+\cdot}$	B_2^+
1	270(100)	269(5)	252(5)	242(22)	–	–	–	152(25)	153(52)	118(40)	121(58)
2	300(100)	299(84)	282(18)	272(5)	271(25)	269(14)	257(62)	152(11)	153(24)	118(12)	121(46)
3	284(100)	283(12)	–	256(4)	255(6)	253(2)	241(18)	152(16)	153(6)	132(36)	135(7)
4	284(100)	283(18)	266(3)	256(11)	255(25)	253(3)	241(18)	166(16)	167(19)	118(16)	121(12)
5	314(100)	313(92)	296(16)	286(80)	285(32)	283(22)	271(85)	152(11)	153(13)	132(23)	135(37)
6	314(100)	313(85)	296(18)	286(10)	285(22)	283(13)	271(54)	166(8)	167(18)	118(8)	121(44)
7	298(100)	297(4)	280(2)	270(8)	269(18)	267(2)	255(18)	166(16)	167(8)	132(21)	155(24)
8	328(100)	327(85)	310(23)	300(9)	299(22)	297(18)	285(92)	166(11)	167(20)	132(17)	155(41)

[a] 1=apigenin; 2=3-methyl-kaempferol; 3=4′(*o*)methyl-apigenin; 4=7(*o*)methyl-apigenin; 5=3,4′-dimethyl-kaempferol; 6=3,7-dimethyl-kaempferol; 7=7,4′-di(*o*)methyl-apigenin; 8=3,7,4′-trimethyl-kaempferol.

Taking into consideration these fragments and analyzing the data shown in Table 1, compounds 1, 3, 4 and 7 can be considered as flavones and compounds 2, 5, 6 and 8 as flavonols.

Substitution of the A- and B-rings, in flavones, can be detected by examining the m/z value for the $A_1^{+\cdot}$ and $B_1^{+\cdot}$ fragments, respectively [9]. In flavones, these fragments are m/z 120 and 102, respectively; in compounds 1 and 3, $A_1^{+\cdot}$ ion is 32 amu higher, that is, m/z 152 instead of 120, indicating that there are two additional oxygen atoms in the A-ring. In compounds 4 and 7 ($A_1^{+\cdot}$, m/z 166) an additional methyl group seems to be present in the A-ring. Similarly, B-ring substitution can be detected; in compounds 1 and 4, $B_1^{+\cdot}$ has a m/z value of 118 instead of 102, thus, an additional oxygen atom seems to be present; in compounds 3 and 7, an additional methyl group also seems to be present ($B_1^{+\cdot}$, m/z 132). With this information, the compounds were located in the NIST (National Institute of Standards and Technology) database [12] and were confirmed using pure standards. As a result, compounds 1, 3, 4 and 7 were identified as apigenin, 4′(*o*)methyl-apigenin, 7(*o*)methyl-apigenin and 7,4′di(*o*)methyl-apigenin, respectively.

Two of the most important diagnostic fragments from flavonols are $[A+1]^+$ and $B_2^{+\cdot}$, and substitution of the A- and B-rings can be deduced by analyzing the m/z values of these fragments [9]. In flavonols, these fragments basically have m/z values of 121 and 105, respectively; in the spectra of compounds 2 and 5, $[A+1]^+$ has an m/z value of 153 instead of 121, indicating that there are two additional oxygen atoms on the A-ring. In compounds 6 and 8, the fragment at m/z 167 indicated an additional methyl group. The substitution patterns of the B-ring were deduced in a similar manner; $B_2^{+\cdot}$ at m/z 121 instead of 105 indicated that there was an additional oxygen atom in the B-ring of compounds 2 and 6; fragments at m/z 135 indicated an additional methyl group in the B-ring of compounds 5 and 8. Using this information, the compounds were located in the NIST database and confirmed using pure standards. As a result, compounds 2, 5, 6 and 8 were identified as being 3-methyl-kaempferol, 3,4′-dimethyl-kaempferol, 3,7-dimethyl-kaempferol and 3,7,4′-trimethyl-kaempferol, respectively.

In summary, the results have shown that LC–PB-MS is a powerful tool for rapid and reliable peak identification with small amounts of plant material, avoiding unnecessary and costly isolation of trivial compounds. Mass spectra of flavonoids showed exactly the same patterns as those obtained with pure compounds by direct solid probe insertion.

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