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Thermospray liquid chromatography–mass spectrometry of flavonol glycosides from medicinal plants

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ABSTRACT

High-performance liquid chromatography interfaced with thermospray mass spectrometry is described for the identification of various flavonol glycosides from *Ginkgo biloba*, *Calendula officinalis* and *Tilia cordata*. Thermospray ionization gave parent species with few and diagnostic fragment ions, thus allowing structure elucidation as well as discrimination between different glycosylation sites.

INTRODUCTION

Phytochemical preparations of medicinal plants are commercially available and used medically for different purposes. TLC and HPLC are generally applied for their analysis. However, identification problems arise when reference standards are not available, and this is often the case for plant extracts. When screening flavonoid glycoside-containing drugs, HPLC with "on-line" ultraviolet detection is normally applied to obtain preliminary information on the analytes' structure [1]. Moreover, semipreparative isolation of unknown flavonoid glycosides followed by hydrolysis and liquid or gas chromatography of the resulting aglycones and sugars provides further data to aid identification [2]. Nevertheless, this approach is time-consuming and laborious. On the contrary, LC–MS represents a fast and reliable method to analyse these non-volatile compounds. Of the different modes, thermospray (TSP) using ammonium acetate as buffer provides mass spectra of flavonoid glycosides nearly identical to those obtained by D/chemical ionization–MS using ammonia [3]. This "soft-ionization" technique produces molecular ions suitable for mass measurements and limited fragment ions, from which the aglycone and the sugar moiety can be determined [4,5]. Extending our study [6] on methods for plant extracts analysis, TSP–MS has been applied to flavonol di- and triglycosides from *Ginkgo biloba*, *Calendula officinalis* and *Tilia cordata* (Fig. 1), and the results are presented in this paper.

EXPERIMENTAL

Materials

Ginkgo biloba leaves, *Calendula officinalis* flowers and *Tilia cordata* leaves were obtained

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PEAK	COMPOUND	R_1	R_2	R_3
I	K-3-O- 6-(P-COUM)-GLU -RHA	H	H	6-(P-COUMAROYL)-GLUCOSYL-RHAMNOSIDE
II	Is-3-O-2^G-RHA-RUTINOSIDE	RHAMNOSIDE	OCH_3	2^G-RHAMNOSYLRUTINOSIDE
1	Q-3-O-GLU-7-O-RHA	RHAMNOSIDE	OH	GLUCOSIDE
2	K-3-O-GLU-7-O-RHA	RHAMNOSIDE	H	GLUCOSIDE
3	Q-3,7-DI-O-RHA	RHAMNOSIDE	OH	RHAMNOSIDE
4	K-3,7-DI-O-RHA	RHAMNOSIDE	H	RHAMNOSIDE

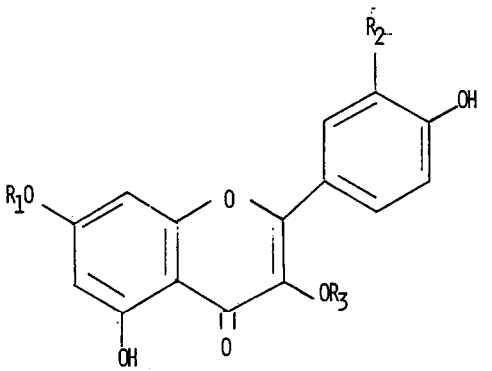

Fig. 1. Structures of the investigated flavonol glycosides. K = kaempferol; Is = isorhamnetin; Q = quercetin.

from Milanfarma)Milan, Italy) and Galke (Gittelde/Harz, Germany). Kaempferol-3-O-[6‴-O-(*p*-coumaroyl)-α-D-glucopyranosyl-(1→4)-α-L-rhamnoside] (I) and isorhamnetin-3-O-2^G-rhamnosyl-rutinoside (II) were isolated from a purified extract of *Ginkgo biloba* [7] and *Calendula officinalis* [8], respectively.

Sample preparation

Compounds I and II were dissolved in methanol (100 μg/ml) and 10 μl were flow injected into the MS apparatus.

Powdered *Tilia* leaves (2 g) were suspended in 50% methanol (20 ml) and left overnight at room temperature. After filtration, the solution was evaporated to dryness *in vacuo* and the residue dissolved in methanol (2 ml). A 20-μl aliquot was injected into the HPLC column.

HPLC conditions

The HPLC apparatus consisted of a Model HP 1090 chromatograph equipped with a Model 1040 photodiode-array detector (Hewlett-Packard, Waldbronn, Germany). The column was an Aquapore RP-300 (4.6 × 220 mm, Applied Biosystems, San José, CA, USA) and the eluents were 2-propanol–tetrahydrofuran–water (10:5:85) and 2-propanol–tetrahydrofuran–ammonium acetate pH 4.5 (10:5:85). The flow-rates were 1.8 and 1.2 ml/min, respectively.

Mass spectrometry

A Hewlett-Packard 5989 mass spectrometer was used together with a Hewlett-Packard thermospray LC–MS interface. The ion source temperature was 220°C, and the vaporizer temperature was held at 100°C. The temperature of the aerosol in the source's jet chamber was 220°C. Full-scan spectra in the range m/z 260–800 in the positive-ion (PI) mode were recorded, except for II, which was taken in the negative-ion mode. Polypropylene glycol was used for mass calibration.

RESULTS AND DISCUSSION

Flavonol glycosides are thermolabile compounds, and the ability to detect their pseudomolecular ions depends on the vaporizer temperature. Using quercetin-3-O-galactoside as a model compound, a relatively high pseudomolecular ion (m/z 465) was obtained by setting the vaporizer temperature at 100°C, although the ion corresponding to the aglycone (m/z 303) produced the base peak of the spectrum. Thus, this vaporizer temperature was chosen for all experiments. The mass spectra of I from *Ginkgo biloba* and II from *Calendula officinalis* confirmed the fragmentation behaviour shown by quercetin-3-O-galactoside. In the PI mass spectrum of I, in addition to the [M + H] ion (m/z 742), fragments that are characteristic of the deacylated flavonoid disaccharide (m/z 433) and of the aglycone (m/z 287) are present (Fig. 2). The fragmentation in the negative-ion mode of the trisaccharide II from *Calendula officinalis* is shown in Fig. 3. The molecular ion [M − H] (m/z 769), the fragment ions originated by loss of the first rhamnose (m/z 623), of the second rhamnose unit (m/z 477) and of glucose to yield the aglycone (m/z 315) are present. To our knowledge, these results represent the first evidence of the potential of TSP for the identification of flavonol acylated diglycoside and flavonol triglycosides.

Owing to the presence in *Tilia cordata* of flavonol derivatives differing in the position of glycosylation, such as quercetin-3-O-glucoside-7-O-rhamnoside (1), kaempferol-3-O-glucoside-7-O-rhamnoside (2), quercetin-3,7-O-dirhamnoside (3) and kaempferol-3,7-O-dirhamnoside (4), it has been interesting to evaluate the data achievable by LC–TSP. Fig. 4 shows a typical reversed-phase chromatogram of an extract of *Tilia cordata* leaves, using 2-propanol–tetrahydrofuran–water (10:5:85) at a flow-rate of 1.8 ml/min. The products of interest were peaks 1–4, whereas peaks 5–8 were not considered since they can be easily identified by co-chromatography with standards and "on-line" UV spectroscopy [7]. For LC–MS analysis of the glycosides 1–4 water in the eluent was replaced by

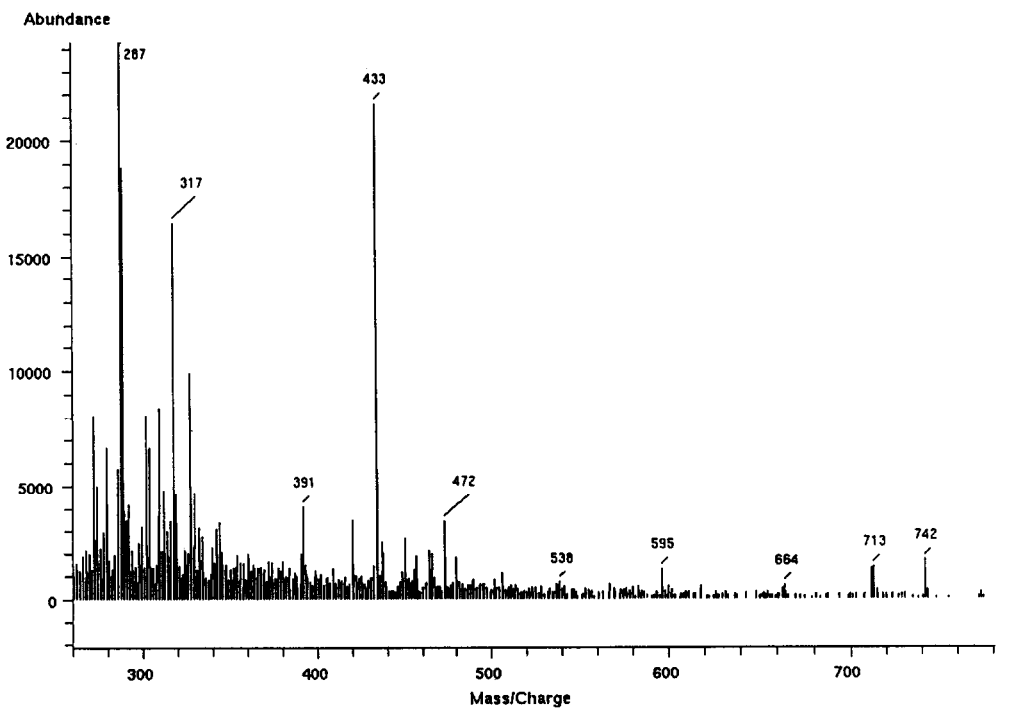

Fig. 2. Mass spectrum of kaempferol-3-O-(6‴-O-*p*-coumaroyl)-glucosyl-rhamnoside (I) from *Ginkgo biloba*.

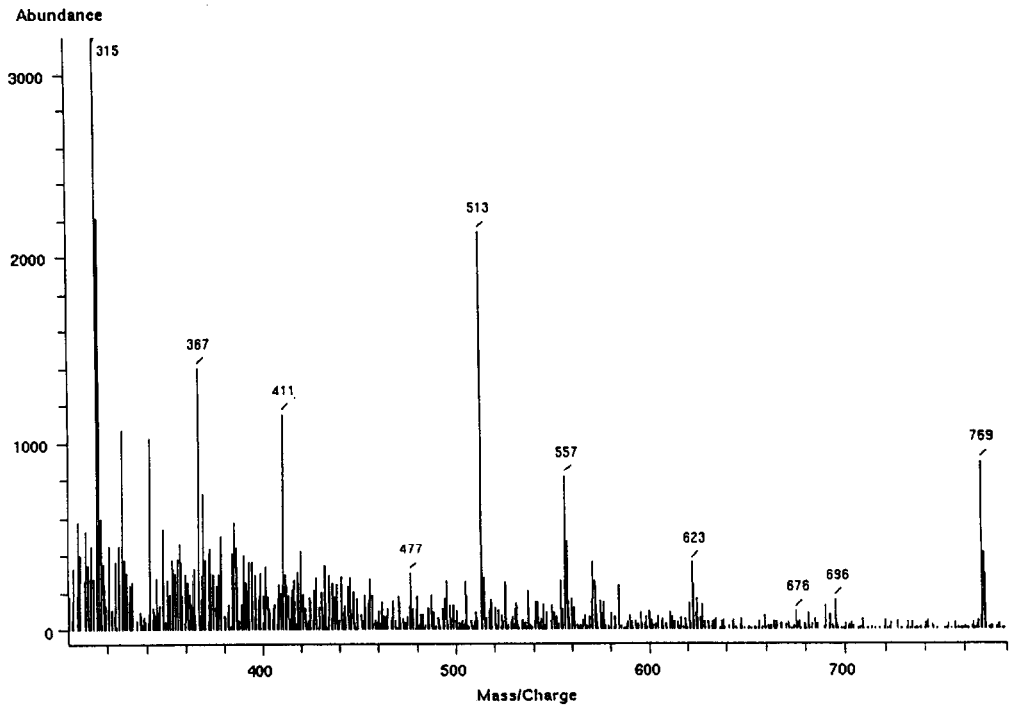

Fig. 3. Mass spectrum of isorhamnetin-3-O-2-rhamnosyl-rutinoside (II) from *Calendula officinalis*.

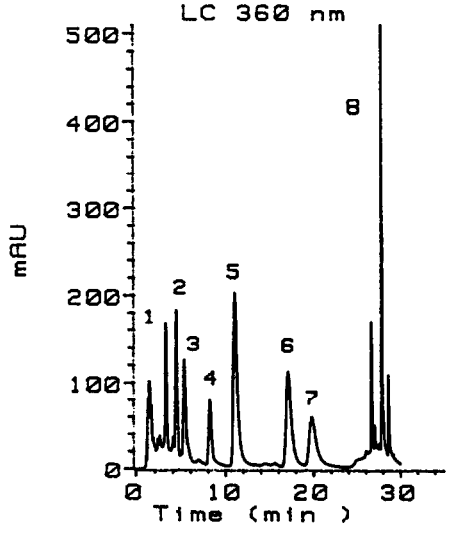

Fig. 4. HPLC of *Tilia cordata*. Column: Aquapore RP-300 (4.6 × 220 mm). Eluent: 2-propanol–tetrahydrofuran–water (10:5:85). Flow-rate: 1.8 ml/min. Peaks: 1–4, see Fig. 1; peak 8 = tiliroside.

0.08 *M* ammonium acetate and the flow-rate was reduced to 1.2 ml/min. After the elution of these peaks, the mobile phase was gradually changed to 100% methanol to rinse the column for a new run. "On-line" photodiode-array detection indicated clearly that compounds 1 and 3 and 2 and 4 was quercetin and kaempferol derivatives, respectively (Fig. 5). Subsequent TSP–MS gave further data (Table I) useful for the identification of these glycosides. As expected, peaks 2 and 3 yielded low protonated molecular ions, both the fragments derived from the parent ion by loss of the 3-O-glucose or the 7-O-rhamnose, and intense aglycone ions. On the contrary, the mass spectra of peaks 2 and 4 presented, together with the small molecular ions, only one intermediate fragment originated by removal of rhamnose from the 3- or 7-position, and abundant aglycone ions. As examples, the spectra of 2 and 4 are shown in Figs. 6 and 7. From these data peaks 1, 2 and 4 can be confirmed as the

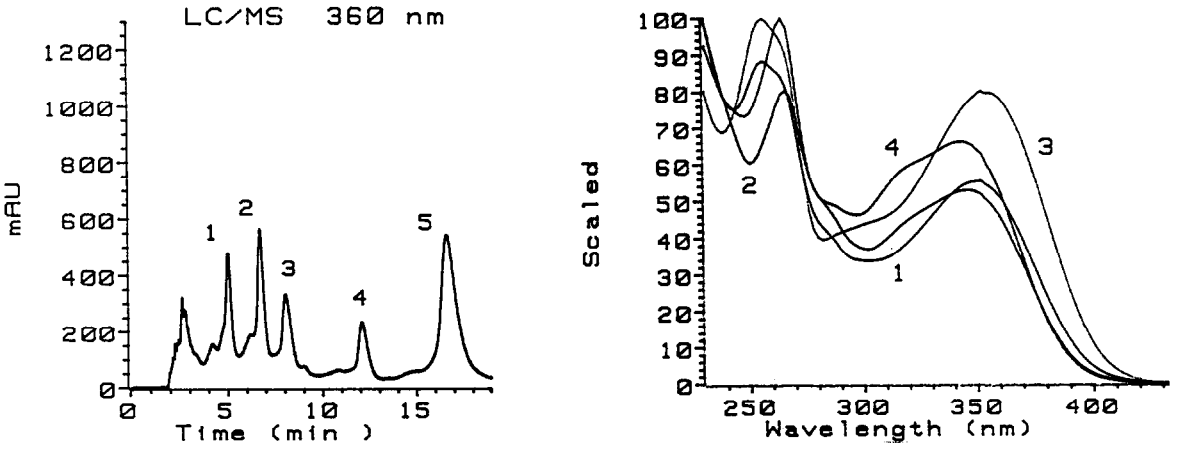

Fig. 5. HPLC of *Tilia cordata* for LC–MS analysis. Column: Aquapore RP-300 (4.6 × 220 mm). Eluent: 2-propanol–tetrahydrofuran–0.08 *M* ammonium acetate pH 4.5 (10:5:85). Flow-rate: 1.2 ml/min. Peaks: 1–4, see Fig. 1; peak 5 = isoquercitrin.

TABLE I

MAIN IONS IN THE TSP SPECTRA OF THE GLYCOSIDES 1–4 FROM *TILIA CORDATA*

		MW	$M+H^+$	Q-glu + H^+	Q-rha + H^+	K-glu + H^+	K-rha + H^+	$Q+H^+$	$K+H^+$
Q-3-glu-7-rha	(1)	610	611	465	449			303	
K-3-glu-7-rha	(2)	594	595			449	433		287
Q-3,7-di-rha	(3)	594	595		449			303	
K-3,7-di-rha	(4)	578	579				433		287

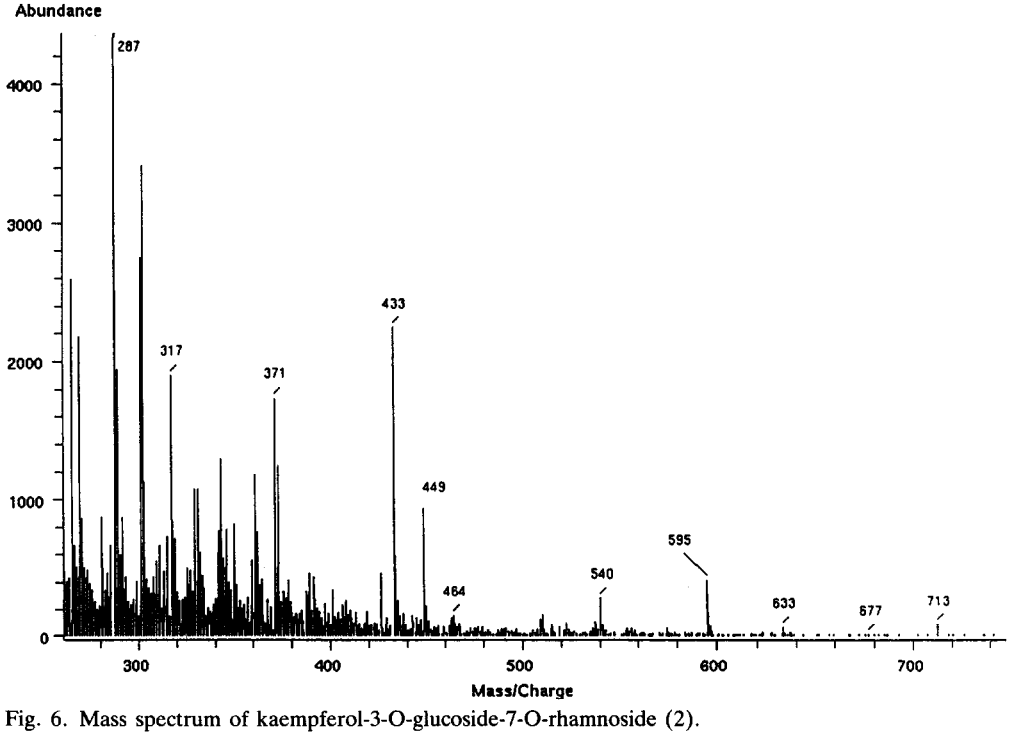

Fig. 6. Mass spectrum of kaempferol-3-O-glucoside-7-O-rhamnoside (2).

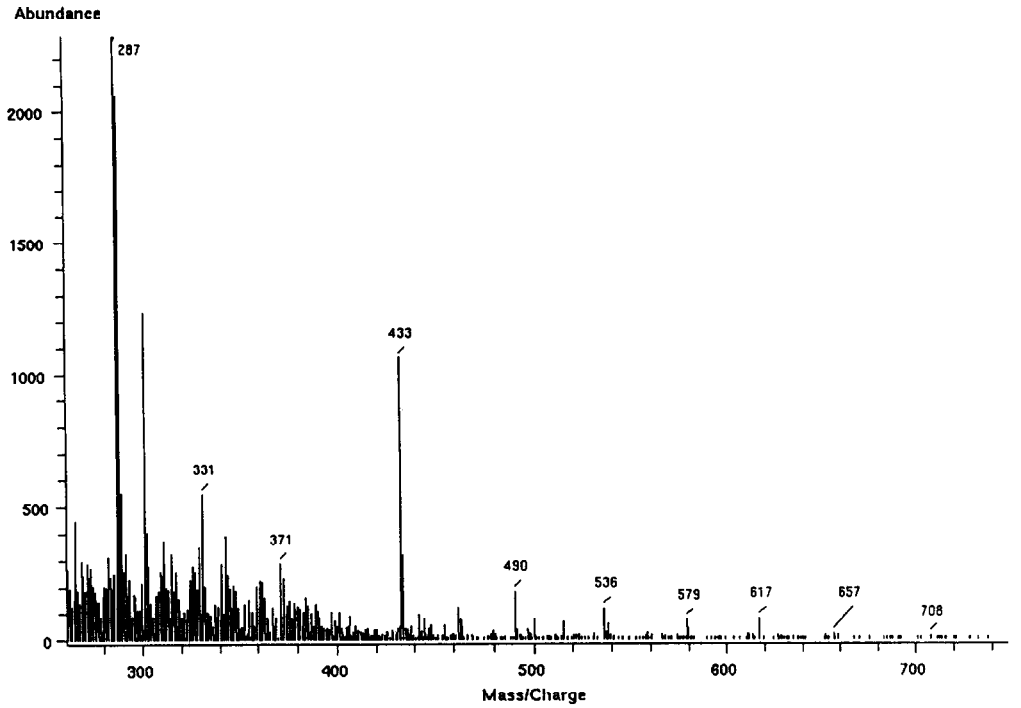

Fig. 7. Mass spectrum of kaempferol-3,7-O-dirhamnoside (4).

previously reported flavonol glycosides [8], while peak 3 can reasonably be assumed to be the analogue quercetin-3,7-O-dirhamnoside.

It may be concluded that TSP is of high value for the analysis of flavonol glycosides occurring in plant extracts, since this ionization technique provides information on molecular ions, sugar sequence and glycosylation site. Moreover, photodiode-array detection can be performed simultaneously, thus obtaining confirmatory data based on ultraviolet spectra.

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