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Identification of Apigenin Metabolites in Rat Liver Perfusate by Column Switching High Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry

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Summary. A selective column switching high performance liquid chromatographic method has been used for the simultaneous determination of apigenin and metabolites in rat liver perfusate. This new method clearly separated apigenin and the metabolites with high resolution. The structures of metabolites were proposed based on selective ion recording and full scan analysis by electrospray ionization mass spectrometry.

Keywords. Endocrine disrupters; Apigenin; Metabolites; HPLC; Column switching; Electrospray.

Introduction

Apigenin is recognized in traditional or alternative medicine for its pharmacological activity. Human exposure to apigenin occurs primarily through the consumption of chamomile and through its presence as a glycoside in many fruits and vegetables including mint, parsley, and celery [1, 2]. In recent years, concerns have been voiced about the presence of compounds in the environment that affect the endocrine and other hormonal systems. These compounds, commonly referred to as ''endocrine disrupters'', pose potential risks to humans, wildlife and the ecosystem. Recent studies highlighted that apigenin could act as an endocrine disrupter by interacting with estrogen receptors [3, 4], thereby preventing the action of natural hormones

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and, by affecting the biosynthesis, thus, altering the concentration of these hormones [5]. Several HPLC methods are described in literature for the analysis of flavonoids in various biological matrices. Column switching assays have the advantages of avoiding off-line procedures and minimize loss that might occur during sample clean-up procedures.

In this paper, in order to expand our knowledge about endocrine effects of apigenin, we have adapted a column switching high performance liquid chromatography/electrospray ionization mass spectrometry to investigate the *ex-vivo* metabolism of apigenin.

Results and Discussion

The operation in SIR modes allies excellent sensitivity with high specificity, as only specific ions are monitored. Metabolites can usually be detected by observing shifts in their masses relative to the parent compound. Experiments were carried out to identify metabolites as glucuronide, sulfate, and methylated or hydroxylated products. Chromatograms in Fig. 1 show the results of SIR experiments. These experiments, carried out with negative electrospray ionisation, clearly demonstrated the presence of monoglucuronides ($m/z = 445$), monosulfates ($m/z = 349$), and a monohydroxylated derivative ($m/z = 285$) which could be attributed to luteolin by comparison with an authentic reference. One should note that neither diglucuronides ($m/z = 621$), sulfoglucuronide conjugates ($m/z = 525$) nor methylated derivatives ($m/z = 283$) were detected by HPLC/MS. The findings were based

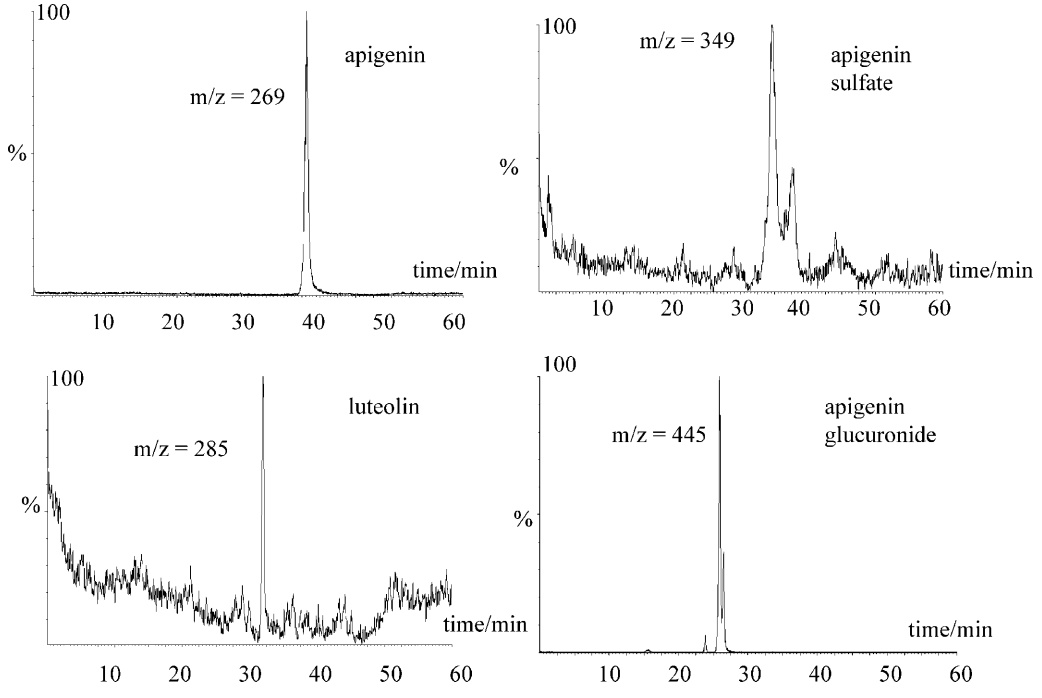

Fig. 1. Single ion recording chromatograms

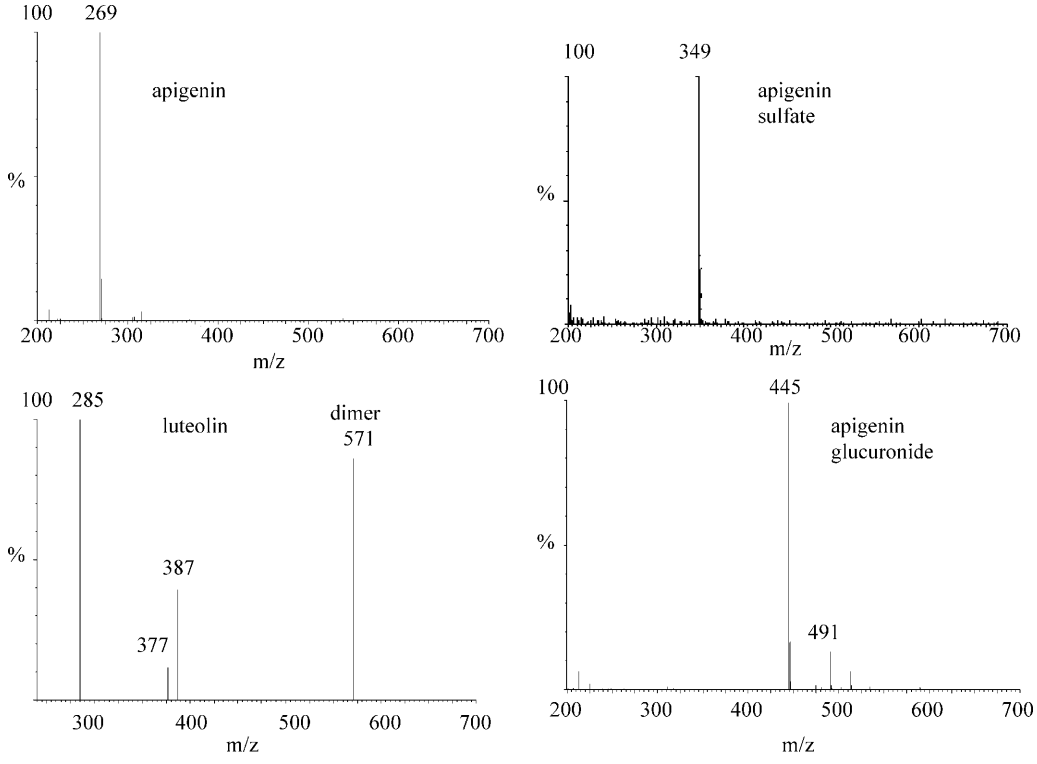

Fig. 2. Electrospray ionization mass spectra of apigenin and metabolites

on full scan data as shown is Fig. 2. Unfortunately, this work could not lead to the identification of regioisomers formed by liver perfusion.

These results are in accordance with those previously published since the main metabolite after *in vitro* biotransformation by rat liver microsomes was identified as luteolin by *Nielsen et al.* [6]. However, *Galijatovic et al.* [7] characterized a glucuronide and a sulfate conjugate after incubation of apigenin in Hep G2 cells. This is not surprising since flavonoids may preferentially undergo glucuronidation and sulfation [8–10].

In conclusion, sample enrichment by column-switching greatly improved the resolution of the method. Direct injection of perfusate failed to demonstrate the presence of luteolin. This study provides direct evidence that apigenin is preferentially glucuronidated and sulfated versus oxidation in perfusates. Biological activities of metabolites are currently under investigation.

Experimental

Apigenin was purchased from Extrasynthèse (Genay, France). HPLC Grade acetonitrile and formic acid were obtained from Merck Eurolab (Gradignan, France). Purified water was obtained by a Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA).

Livers were isolated as described by *Teyssier et al.* [11]. Briefly, the perfusion medium consisted of *Krebs-Henseleit* bicarbonate buffer *pH* 7.4 containing glucose (10 mM final concentration). After a single-pass equilibration period the experiments were performed with recirculation of the perfusate. The reservoir (150 cm^3) contained 0.1 mM of apigenin. Samples (0.7 cm^3) were collected at 120 min

Table 1. Chromatographic conditions

Time	% mobile phase (v/v)		Column switch
(min)	A[a]	B[b]	position of valve
0	90	10	1
5	90	10	2
10	90	10	2
30	70	30	2
45	70	30	2
50	90	10	2
60	90	10	1

[a] A = water:formic acid = 99.5:0.5
[b] B = acetonitrile:formic acid = 99.5:0.5

following initiation of flavonoid perfusion. The viability of the liver was assessed by visual appearance and measuring lactate dehydrogenase activity in the perfusate.

The high performance liquid chromatography (HPLC) was performed with a Waters Alliance system (Milford, MA, USA) including 2690 pumps and a 996 photodiode array detector (DAD). A single quadrupole Waters LCZ Platform (Milford, MA, USA) equipped with an ESI source operated in negative ionization mode was used for the work described herein. Mass spectra were acquired in selective ion recording (SIR) mode or full scans from $m/z = 200$ to 700 at 1 scan/s using the following conditions. ESI capillary voltage: 3.50 kV; desolvation temperature: 150°C; flow of desolvation gas: 400 dm^3/h; fragmentor voltage: 20 V.

The column switching procedure used in this study has been previously reported by *Nielsen et al.* [12]. Briefly, a six-port valve was programmed to switch from position 1 (precolumn) to position 2 (precolumn + analytical column). The precolumn was filled with Kromasil C18 adsorbent (3.5 µm particle size, 20×2 mm) and the analytical column with Nucleosil C18 (3 µm particle size, 125×2 mm) on which metabolites which had been retained on the precolumn were eluted. Table 1 shows in detail the chromatographic conditions for the column switch HPLC methodology developed.

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