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Note

Evaluation of the moving belt as an interface for the high-performance liquid chromatographic–mass spectrometric analysis of the flavonoid aglycones

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The moving belt[1] is a transport interface that makes possible the direct coupling of high-performance liquid chromatography (HPLC) and mass spectrometry (MS), obtaining true electron impact (EI) and chemical ionization (CI) mass spectra with free selection of reagent gases[2–4].

Although EI and/or CI mass spectra alone can hardly establish completely the structure of a given flavonoid aglycone, they can always determine the molecular weight of these compounds and almost always the distribution of substituents between the A- and B-rings (see the structure insert in Fig. 1)[5–11]. The mass spectra of the flavonoids are also quite characteristic and can differentiate many isomers: compare, for instance, the spectra of quercetin and morin in Table I. Further, detailed EI mass spectrometric studies are available of the flavonoids that permit assignement of complicated substitution patterns[12–14]. Thus, with the combination of HPLC/UV–visible techniques[15], HPLC–EI-MS and HPLC–CI-MS analysis would be enough for the complete identification of many flavonoid aglycones, if a system for such HPLC–MS analysis was available.

Previously, we have studied some phenolic compounds by HPLC–MS using the moving belt as interface[16–22]. Here we report a study of the suitability of the belt for the HPLC–MS analysis of representative compounds of four flavonoid aglycone classes: flavones, flavonols, flavanones and flavanonols, covering an extensive range of polarities.

EXPERIMENTAL

A VG Analytical moving belt (which provides sample flash evaporation directly into the ion source) interfaced to a VG Analytical double focusing 7070E mass spectrometer, operated at low resolution (R_s = 1000) and equipped with an EI–CI ion source has been used.

A polyimide (Kapton®) belt at 1.6 cm/s was used. Conditions: infrared solvent heater, 150°C (indicated); sample evaporator temperature, 200°C (indicated); ion

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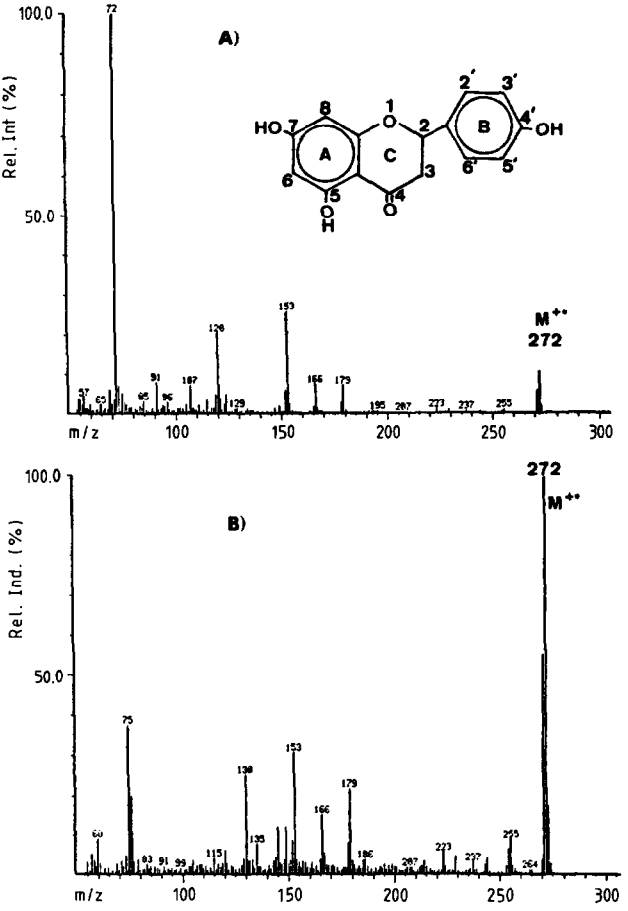

Fig. 1. EI mass spectra of naringenin obtained using the moving belt as inlet system: (A) and (B), 2 and 4 μg of the compound spotted on the belt, respectively.

source temperature, 200–210°C in EI and CI mode; ion source pressures, (a) $2 \cdot 10^{-6}$ Torr in EI mode that increased to $6 \cdot 10^{-6}$–$8 \cdot 10^{-6}$ Torr when working with LC on line and (b) $4 \cdot 10^{-5}$ Torr in CI mode (methane as reagent gas); electron energy and emission current, 70 eV and 200 μA, respectively. The scan rates and mass ranges were (a) 2 s per decade and 35 to 500 a.m.u. for the spotting experiments in the EI mode; and (b) 3 s per decade and 90 to 500 a.m.u. for the HPLC–MS analysis, detection limit and spotting studies in the CI mode.

The chromatographic system consisted of Waters 6000 A pumps, an M660 solvent programmer and an M441 UV absorbance detector (254 nm). The column was a Hypersil ODS 2 (10 cm × 4.6 mm I.D.) with a 3-μm particle diameter.

The solvent used was methanol–5% aqueous acetic acid (60:40, v/v) at a flow-rate of 0.9 ml/min directly fed on the belt with an electrically heated spray deposition device.

Flavonoid standards were obtained from commercial suppliers (Fluka, Madaus, Sarsyntex) or as described in refs. 23 and 24.

TABLE I

EI AND METHANE CI MASS SPECTRA OF SELECTED FLAVONOID AGLYCONES OBTAINED SPOTTING THE COMPOUNDS IN METHANOLIC SOLUTION DIRECTLY ONTO THE MOVING BELT INTERFACE

Only structurally significative peaks are quoted (see analytical conditions in the Experimental section).

Compound and substitution pattern	*Molecular weight*		*m/z (% relative intensity)*
Flavones			
Flavone	222	EI	222(100), 221(34), 194(30), 165(7), 120(42), 97(4), 92(7)
		CI	251(8), 224(15), 223(100), 222(6)
Chrysin 5,7-$(OH)_2$	254	EI	254(100), 253(16), 226(31), 152(49), 124(36), 105(7), 102(11), 96(15)
		CI	283(12), 256(21), 255(100), 254(26), 237(6), 153(18)
Apigenin 5,7,4′-$(OH)_3$	270	EI	270(100), 269(12), 242(15), 124(23), 121(31)
		CI	299(12), 272(22), 271(100), 270(19)
Acacetin 5,7-$(OH)_2$-4′-OCH_3	284	EI	284(100), 283(10), 256(4), 241(10), 152(8), 132(21), 124(5)
		CI	313(5), 286(19), 285(100), 284(13), 257(4)
Luteolin 5,7,3′,4′-$(OH)_4$	286	EI	286(100), 285(9), 258(17), 229(9), 153(67), 137(6), 134(21), 124(18), 96(9)
Xanthomicrol 5,4′-$(OH)_2$-6,7,8-$(OCH_3)_3$	344	EI	344(61), 343(3), 329(100), 314(6), 301(4), 211(24), 183(16), 118(9)
		CI	373(36), 346(72), 345(100), 344(82), 343(9), 329(33), 315(5), 314(4)
Cirsimaritin 5,4′-$(OH)_2$-6,7-$(OCH_3)_2$	314	EI	314(98), 313(23), 299(100), 285(25), 271(33), 181(29)
		CI	343(8), 316(21), 315(100), 314(17), 299(6), 285(9), 271(6), 265(12), 255(21), 205(12)
Diosmetin 5,7,3′-$(OH)_3$-4′-OCH_3	300	EI	300(100), 299(2), 284(9), 271(5), 257(18), 229(12), 153(12), 148(6), 133(8), 105(3)
		CI	329(9), 302(15), 301(100), 300(19), 285(7), 283(5), 153(4)
Flavonols			
Kaempferol 3,5,7,4′-$(OH)_4$	286	EI	286(100), 285(25), 258(9), 229(7), 213(6), 153(5), 143(4), 121(10)
		CI	315(4), 288(19), 287(100), 286(20)
Quercetin 3,5,7,3′,4′-$(OH)_5$	302	EI	302(100), 301(24), 273(13), 257(9), 245(9), 229(11), 153(29), 137(43), 109(22)
		CI	331(13), 304(23), 303(100), 302(32)
Morin 3,5,7,2′,4′-$(OH)_5$	302	EI	302(99), 301(7), 286(54), 285(100), 153(32), 137(16)
		CI	304(18), 303(100), 302(11)
Flavanones			
Naringenin 5,7,4′-$(OH)_3$	272	EI	272(100), 271(56), 254(7), 244(4), 179(22), 166(15), 153(31), 120(6)
		CI	301(6), 274(20), 273(100), 272(16), 265(7), 255(7), 179(31), 153(35), 147(7)

TABLE I (*continued*)

Compound and substitution pattern	*Molecular weight*		*m/z (% relative intensity)*
Eriodictyol 5,7,3',4'-$(OH)_4$	288	EI	288(10), 287(5), 179(21), 166(31), 153(100), 136(76), 123(46), 110(18)
Flavononols			
Taxifolin 3,5,7,3',4'-$(OH)_5$	304	EI	304(18), 286(5), 275(31), 165(17), 153(79), 152(31), 137(30), 123(45)
		CI	306(15), 305(100), 304(4), 289(17), 287(33), 275(15), 259(19), 195(9), 181(9), 153(14)

RESULTS AND DISCUSSION

Polarity and possibility of handling the flavonoid aglycones by the belt

At the moment, no HPLC–MS system or interface is of universal application[3,4] In order to determine which types of flavonoids can be investigated by the VG moving belt, initial studies were conducted with a series of flavonoids (Table I), by spotting solutions of the samples (1–2 μl of a 1-mg/ml solution of each compound in methanol) onto the belt under a variety of source and sample evaporator temperature settings. Flavone, xanthomicrol, cirsimaritin and other apolar flavonoids provided very good EI and CI mass spectra from the moving belt interface and temperature control was not very critical. On the other hand, the more polar and involatile compounds, as luteolin and taxifolin, for instance, were very sensitive with respect to the temperature settings. 200°C for the sample evaporator and 200–210°C for the ion source were the most convenient for most of the flavonoids studied as well in the EI as in the CI mode. However, the belt was unsuitable for the mass spectral analysis of high-polarity and high-molecular-weight compounds as the flavonolignans silybin, silydianin and silychristin. There was not a recognizable molecular ion for these flavonoids using the belt as inlet system.

With regard to the spectral quality, there are two features worthy of consideration. (i) The background contribution to the spectra (even after background subtraction) with the more involatile samples is intense and it often gives an intense peak at *m/z* 149 due to belt plasticizers. This is specially inconvenient for the EI-MS analysis of the flavonoids because of its proximity to the diagnostic peaks at *m/z* 152 and/or *m/z* 153 of many 5,7-dihydroxyflavonoids and a peak at *m/z* 177 that interferes with the diagnostic peak at *m/z* 179, $[M - (B\text{-ring})]^+$, of the 5,7-dihydroxyflavanones. (ii) With some polar compounds, there is also a notable diminution of the relative intensities of the highest mass ions (molecular ion included) compared with the corresponding direct-probe inlet EI mass spectra. This, added to the relatively high background levels in the spectra of these compounds, leads to useful mass spectra but only of medium quality.

Thus, in the given conditions, the moving belt studied is a good EI-as well as CI-MS inlet for the flavonoids of low to medium polarity examined, but limited with respect to high polar and involatile compounds.

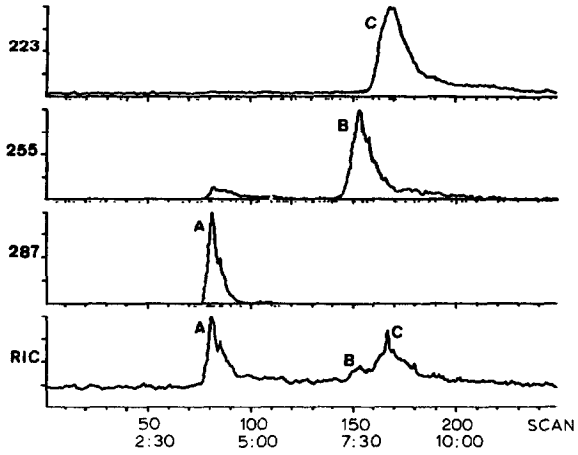

Fig. 2. Methane CI HPLC–MS computer reconstructed total-ion current trace (RIC) and extracted mass chromatograms ($[M + H]^+$ ions) obtained for a mixture of 10 μg of kaempferol (mol.wt. 286; peak A), 3 μg of chrysin (mol.wt. 254; peak B) and 3 μg of flavone (mol.wt. 222; peak C) injected on column (see chromatographic and MS conditions in the Experimental section).

Detection limits and HPLC–MS on line of the flavonoid aglycones

The mass spectra obtained using the moving belt are dependent on the quantity of sample spotted on the surface[21,25]. In our case, spotting 2 or 4 μg of naringenin on the belt has a dramatic effect on the relative intensity of the molecular ion, as it is shown in Fig. 1. Because of this, it is worthy to study the practical detection limits of the flavonoid aglycones using this interface at full scan, both spotting the samples directly on the belt and in true HPLC–MC conditions. The minimum amounts of

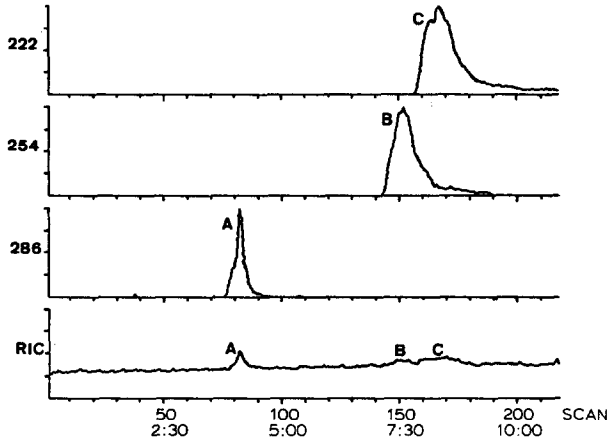

Fig. 3. HPLC–EI-MS computer reconstructed total-ion current trace (RIC) and extracted mass chromatograms ($M^{+\cdot}$ ions) obtained for a mixture of 10 μg of kaempferol (peak A), 3 μg of chrysin (peak B) and 3 μg of flavone (peak C) injected on column (see chromatographic and MS conditions in the Experimental section).

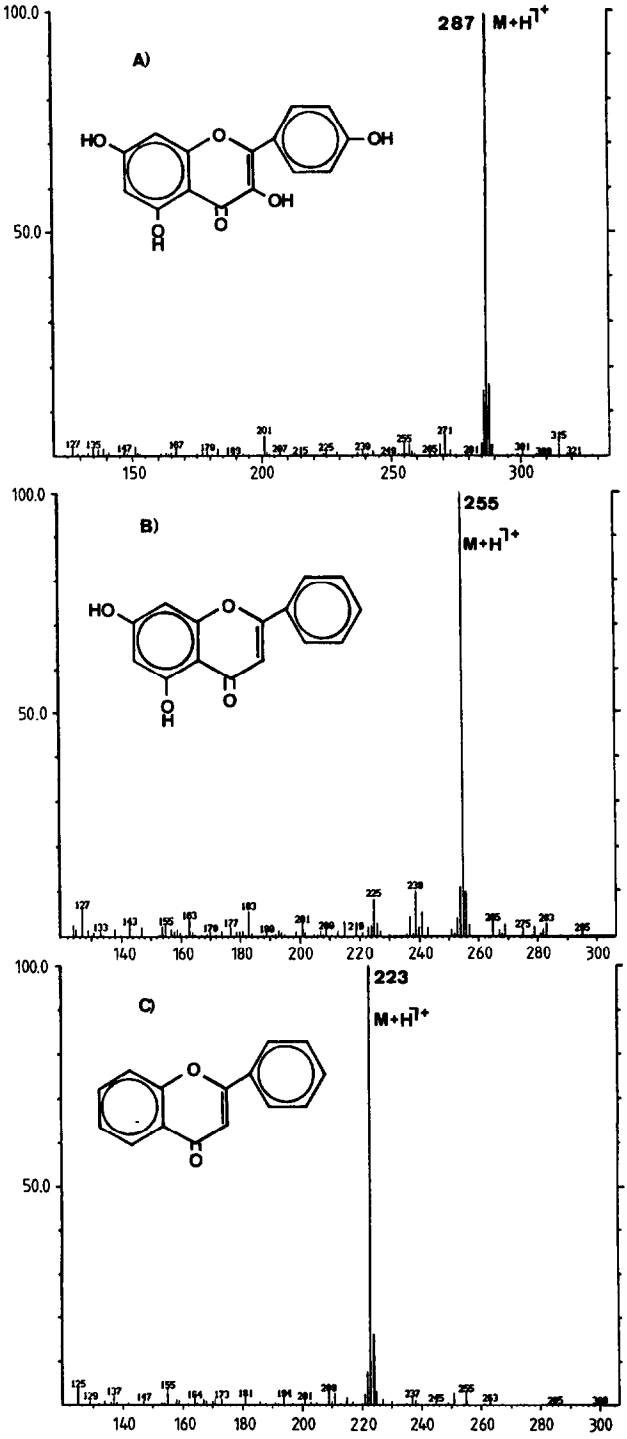

Fig. 4. (A), (B) and (C), methane CI mass spectra of peaks A, B and C, respectively, in the HPLC–MS chromatogram shown in Fig. 2.

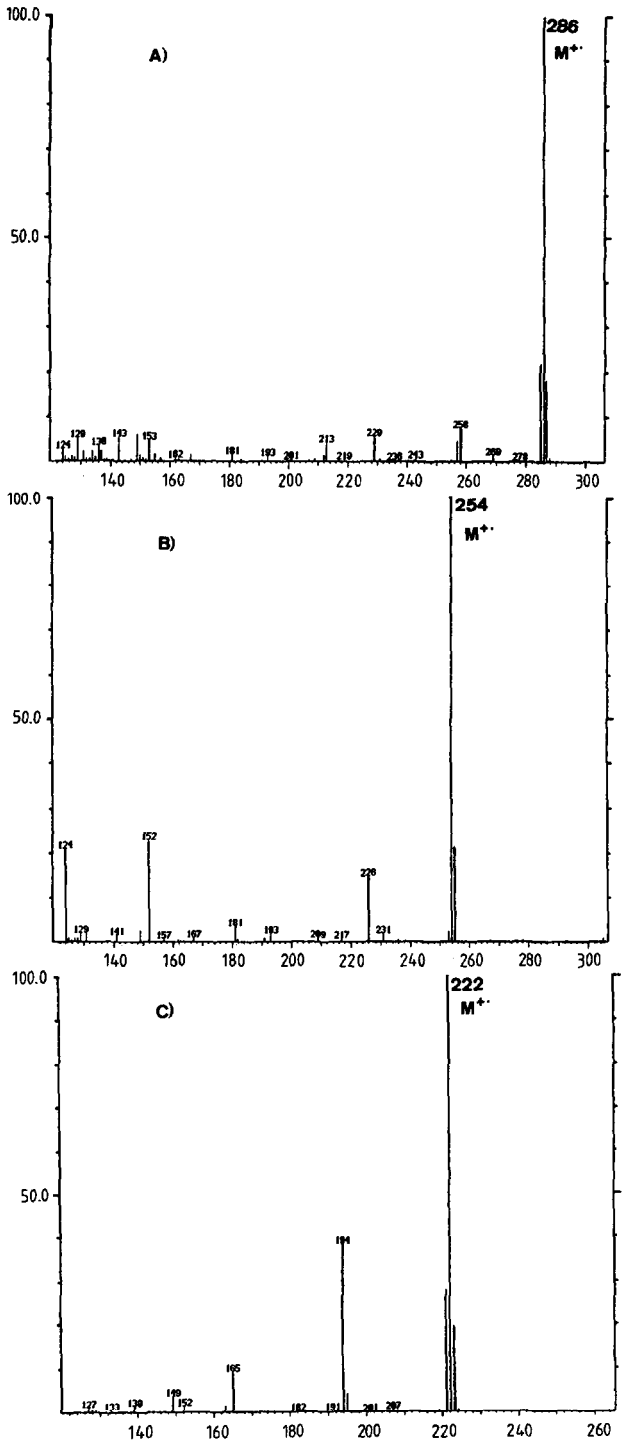

Fig. 5. (A), (B) and (C), EI mass spectra of peaks A, B and C, respectively, in the HPLC–MS chromatogram in Fig. 3.

sample effectively spotted or injected on the column that give an interpretable mass spectrum and comparable to the direct inlet spectrum of the same compound are as follows: flavone, 0.05 μg spotting and 0.1 μg injecting; kaempferol, 0.5 μg spotting and 4.0 μg injecting. For this purpose, we have chosen a flavonoid of low polarity (flavone) and one of medium-high-polarity (kaempferol) in order to cover a reasonable polarity range. Obviously, because of the column dilution effect, when working on line, the limits are higher than when spotting directly onto the belt. Thus, for those compounds which tend to be adsorbed on the belt surface, the amount of sample needed for the detection and structural analysis of the flavonoids injected on column is quite high.

With respect to the HPLC–MS flavonoid mixture analysis using the VG moving belt, as in many HPLC–MS mixture analysis, with this and other systems, the computer reconstructed total ion current traces are poor, even scanning 90 a.m.u. upwards to avoid solvent interferences (Figs. 2 and 3). But, for low molecular weight and/or low polarity flavonoids, the $[M + H]^+$ ion (CI mode) or $M^{+\cdot}$ ion (EI mode) mass chromatograms are acceptable and the spectra of every peak, even for those not well resolved, provide very valuable structural information, as it is shown in Figs. 2–5.

Thus, whereas the detection of single flavonoids and simple mixtures of low-polarity compounds injected on column is possible with the HPLC–MS system used, it is unsuitable for the analysis of complex mixtures including high-polarity and involatile flavonoids as the flavonolignans, for instance.

Perhaps newer belt interface designs[21,26] can overcome some of these drawbacks. On the other hand, given the results obtained with fast atom bombardment (FAB) MS studies of the flavonoid aglycones[27], HPLC–FAB-MS using the moving belt[26,28] could be an useful system for the HPLC–MS analysis of these compounds.

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