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Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments

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Abstract

With 15 flavonoids as test compounds, the analytical performance of four modes of LC–MS, multiple MS (MS^n) and tandem MS operation (atmospheric pressure chemical ionization (APCI), electrospray ionization, positive and negative ionization) was compared for two mass spectrometers, a triple-quadrupole and an ion-trap instrument. Two organic modifiers, methanol and acetonitrile, and two buffers, ammonium acetate and ammonium formate, were used. In general, the use of APCI in the negative ion mode gave the best response, with the signal intensities and the mass-spectral characteristics not differing significantly between the two instruments. The best results were obtained when methanol–ammonium formate (pH 4.0) was used as LC eluent. Under optimum conditions full-scan limits of detection of 0.1–30 mg/l were achieved in the negative APCI mode. Here it needs to be emphasized that up to 2-order response differences were found both between analytes and between modes of ionization. This implies that one should be very cautious when interpreting data on the screening of real-life samples. The main fragmentations observed in the MS^n spectra on the ion-trap, or the tandem MS spectra on the triple-quadrupole were generally the same. The advantage of the former approach is the added possibility to ascertain precursor→product ion relationships.

Keywords: Mass spectrometry; Flavonoids; Glycosides

1. Introduction

In the literature, various groups of workers have devoted considerable attention to the determination of flavonoids in a variety of samples [1–12]. Flavonoids, which are one of the most characteristic classes of compounds in higher plants, comprise a wide group of structurally related compounds with a chromane skeleton and a phenyl substituent in the C2 (flavones) or C3 (isoflavones) position. Structures and abbreviations used in this paper are given in Fig. 1. Flavonoids that have a single C2–C3 bond are called flavanones; with an OH group in the C2 position they are called flavonoles. Flavonoids are usually found in plants as glycosides, i.e. provided with one or more sugar substituents such as galactose, rhamnose or glucose, or glucoside malonate. Because flavonoids are moderately polar, ther-

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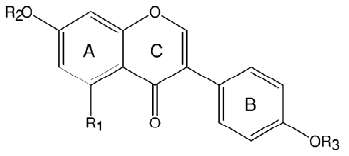
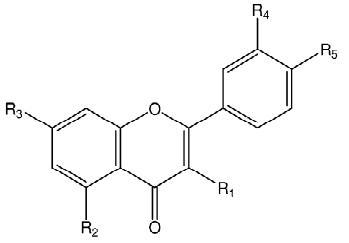
Isoflavones



Name	Abbrev.	R_1	R_2	R_3	MW
Daidzein	D	H	H	H	254
Daidzin	DG	H	7-O-β-D-Gly	H	416
Genistein	G	OH	H	H	270
Genistin	GG	OH	7-O-β-D-Gly	H	432
Formononetin	F	H	H	CH_3	268
Ononin	FG	H	7-O-β-D-Gly	CH_3	430
Biochanin A	B	OH	H	CH_3	284
Sissotrin	BG	OH	7-O-β-D-Gly	CH_3	446

Flavones



Name	Abbrev.	R_1	R_2	R_3	R_4	R_5	C_2-C_3	MW
Hesperedin	HG2	H	OH	7-O-β-D-Glypyr	OH	OCH_3	single	610
Hesperitin	H	H	OH	OH	OH	OCH_3	single	302
Naringin	NG2	H	OH	7-O-β-D-Glypyr	H	OH	single	580
Naringenin-7-glycoside	NG	H	OH	7-O-β-D-Gly	H	OH	single	434
Naringenin	N	H	OH	OH	H	OH	single	272
Rutin trihydrate	RG2	7-O-β-D-Glypyr	OH	OH	OH	OH	double	610
Kaempferol	K	OH	OH	OH	H	OH	double	286

Fig. 1. Structures of the flavonoids studied and their acronyms: Gly, glycoside; Glypyr, glycopyranosyl. Rings and bonds indicated by A, B, C and 1–4, respectively, are discussed in Section 3.5.

molabile compounds, they are best analyzed with reversed-phase LC. Obviously—especially if identification of individual flavonoids is important—advanced hyphenated techniques have to be used, e.g. LC–MS or LC–MS–MS. Various ionization methods, such as thermospray, fast atom bombardment and atmospheric pressure ionization (API), have been applied to determine flavonoids. Today, API-MS methods in particular receive much attention. For the broad class of flavonoids, which comprises aglycons, glycosides and conjugates such as malonates and acetates, both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are used. Good results can be obtained with both techniques, even with ESI, although the flavonoids—especially the aglycons—are not very polar. Selected papers on the determination of flavonoids with LC coupled to positive- or negative-mode APCI and/or ESI are listed in Table 1. Some authors have stated that there are differences in analyte response

Table 1
Selected papers on LC–API-MS(–MS) of flavonoids[a]

Analytes	Sample	LC eluent	API mode	Type of MS	Ref.
Flavonoids	Fresh herbs	MeOH–water, 1% FA	APCI−	Q	[1]
Isoflavones	Soy foods	ACN–water, 0.1% TFA or 0.1% AA	APCI± and IS±	QqQ	[2]
Flavonoids	Red clover	ACN–water, 0.25% AA	ESI±	Q	[3]
Phenolic compounds	Soy, onions	ACN–water, 10%FA	ESI−	Q	[4]
Phenolic compounds	Olives	MeOH–water, 1% AA	ESI±	QqQ	[5]
C-Glycosidic flavonoids	Not applied	ACN–water, 0.5% AA	APCI± and ESI±	IT and Q-TOF	[6]
Flavonoids	Wood pulp, waste water	MeOH–water, 0.5% AA	ESI±	QqQ	[7]
Flavonoids	Urine	MeOH–ACN–water, 0.5% FA	APCI−	Q	[8]
Flavonoid aglycones	Not applied	MeOH–water, 0.1% FA	ESI−	IT	[9]
Flavonoids	Onion, blossom and St. John's wort	ACN–water, 20 m*M* TFA	ESI+	IT	[10]
Flavonoids	Not applied	MeOH–water or ACN–water, 0.1–0.4% FA or 10 m*M* AAc or 0.1% AH, 0.05% TFA	IS±, APPI± and APCI±	QqQ	[11]
Isoflavones	Red clover	ACN–water, 0.2%AA	ESI+	Q	[12]

[a] AA, acetic acid; AAc, ammonium acetate; ACN, acetonitrile; AH, ammonium hydroxide; APPI, atmospheric pressure photoionisation; FA, formic acid; IS, ion spray; IT, ion trap; MeOH, methanol; TFA, trifluoroacetic acid; TOF, time of flight; Q, single quadrupole; QqQ, triple quadrupole.

between the different ionization modes [5], and that the choice of organic modifier (and buffering agent) may also play a role, but so far, only Rauha et al. [11] have recently studied solvent effects on the ionization and detector sensitivity of five flavonoids. However, generally speaking little systematic work has been performed in this area. Since the goal of most studies, including ours, is to monitor target analytes as well as to screen for unknown flavonoids in plants or food, the analyte responses obtained with the various methods play an important role and require due attention.

The aim of the present paper is to compare the analytical performance of the APCI and ESI API techniques, both in the positive and negative mode. This was done for two different mass spectrometers, a triple-quadrupole and an ion-trap instrument, since their differences in design may affect the MS mode

selection. Standard solutions containing 15 selected flavonoids were used to study the differences in analytical performance.

2. Experimental

2.1. Materials

Daidzin, daidzein, genistin, genistein, naringin, naringenin-7-glycoside, naringenin, formononetin and ononin were purchased from Roth (Karlsruhe, Germany), biochanin A and sissotrin from Indofine Chemical (Somerville, NJ, USA), rutin trihydrate and kaempferol from Fluka Chemie (Buchs, Switzerland), and hesperedin, hesperitin, ammonium acetate and ammonium formate from Sigma–Aldrich (Steinheim, Germany). The flavonoids were analyzed in two mixtures (see Section 3.1); their concentration was 20 mg/l in methanol.

Methanol and formic acid were from J.T. Baker (Deventer, The Netherlands), and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, The Netherlands).

2.2. Methods

Analyses were performed on two LC–MS systems. One was a Shimadzu (Princeton, NJ, USA) LC system, consisting of two LC-10A LC pumps, a DGU-14A degasser, a SIL-10AD auto-injector, a SCL-10A system controller unit and a SPD-10A UV detector (set at 250, 265 and 290 nm), coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion-trap mass spectrometer. The other LC–MS system consisted of a HP 1090 Series II LC instrument (Hewlett-Packard, Palo Alto, CA, USA) coupled to a Quattro II triple-stage quadrupole MS (Micromass, Altrincham, UK). Both mass spectrometers had APCI and ESI interfaces. A 250×4.6 mm I.D., 5 μm Zorbax SB-C_{18} column was used for separation on both systems. The LC eluent consisted of a mixture of an organic modifier and an aqueous volatile buffer. Four combinations were tested: either 10 m*M* aqueous ammonium formate (AF) or 10 m*M* aqueous ammonium acetate (AA) buffer (both pH 4.0), and methanol (MeOH) or acetonitrile (ACN). The AF and AA buffer pH values were adjusted by adding formic acid and acetic acid, respectively. The LC gradients for the two flavonoid mixtures are shown in Table 2. The flow was 1.0 ml/min and the injection volume, 10 μl. All solvents were filtered and degassed with helium before use.

APCI and ESI mass spectra were acquired in the positive ion (PI) and negative ion (NI) mode in the range of m/z 150–650. On the ion-trap MS, the capillary temperature for APCI was maintained at 250 °C and the vaporizer temperature at 450 °C. These intermediate values were used because varying the capillary and vaporizer temperatures within the range specified by the manufacturers did not noticeably change the mass spectra. For ESI, the capillary temperature was optimized by direct infusion of a 5-mg/l methanolic solution of genistin while varying the temperature from 200 to 340 °C. The total signal in full-scan acquisition did not vary much with temperature, but at temperatures below 270 °C increasing adduct formation (Na^+ adducts and double ions in the PI mode and formic acid adducts in the NI mode) occurred. Therefore, the

Table 2
Gradient conditions (%, v/v) for eight-compound mixture (A and B) and seven-compound mixture (C and D) with MeOH or ACN, and AF or AA, both at pH 4.0

	Time (min)	% MeOH	% AF or AA
A	0	30	70
	2	40	60
	10–12	55	45
	19–28	80	20
	31	30	70
B	0	20	80
	20	60	40
	22–24	95	5
	29	20	70
C	4	30	70
	9–14	45	55
	17	55	45
	22–25	60	40
	30	30	70
D	0–3	20	80
	10–13	30	70
	20	40	60
	25	20	80

capillary temperature was set at 275 °C. After optimization for all flavonoids, the capillary voltage was set at 4 V for PI ESI, −4 V for NI ESI, 46 V for PI APCI and −12 V for NI APCI. The APCI corona discharge current was 10 μA. The sheath-gas (nitrogen) flow rates for APCI and ESI were 80 and 40 U, respectively, which corresponds to approximately 200 and 20 l/h, and the auxillary gas (nitrogen) flow rate was 20 U for both APCI and ESI. For multiple MS (MS^n) spectra of selected precursor ions, the activation energy was optimized between 20 and 50% in the PI and NI ESI mode by direct infusion of 5-mg/l methanolic solutions of all compounds. For all compounds, the highest total signal in full-scan acquisition was obtained at 30%. For MS^n experiments, higher activation energies were necessary to obtain complete fragmentation (see Section 3.5).

Optimization of the source temperature and cone voltage settings for triple-quadrupole MS was performed in the same way as for the ion-trap instrument. The source and probe temperatures for APCI were maintained at intermediate values of 150 and 400 °C, respectively. For ESI the source temperature was varied between 70 and 140 °C; for temperatures below 80 °C more adduct formation occurred. Therefore, the source temperature used for all analyses was 120 °C for ESI. After optimization for all flavonoids, the cone voltage for ESI and APCI was set at −50 V (NI) or 50 V (PI), while the probe voltage was set at 3.5 V for PI ESI and −3.0 V for NI ESI. The APCI corona discharge was 5 kV. Sheath-gas (nitrogen) flow rate was 150 l/h for APCI and nebulizing gas (nitrogen) was 15 l/h for ESI. Drying gas (nitrogen) flow rate was 200 l/h for ESI and 300 l/h for APCI.

With both instruments, for APCI the LC flow was directed into the mass spectrometer without stream splitting, while for ESI the LC flow was split and 0.2 ml/min was sent to the mass spectrometer.

3. Results and discussion

3.1. LC–UV

In order to obtain a complete separation of the 15 selected flavonoids, long gradient LC runs would be needed, since the flavonoid glycosides elute at closely similar retention times. Therefore, the 15 compounds were divided into two groups, which of course doubled the number of runs, but shortened the total time of analysis. Separation of the eight-compound mixture was achieved under the gradient conditions given in Table 2A,B. For the seven-compound mixture a slightly different gradient had to be used (Table 2C,D).

In Fig. 2 the UV responses of the 15 flavonoids are shown for the four buffer–modifier combinations tested. The response is expressed as peak area, recorded at 250 (FG, F, DG, D), 265 (BG, K, B, GG,

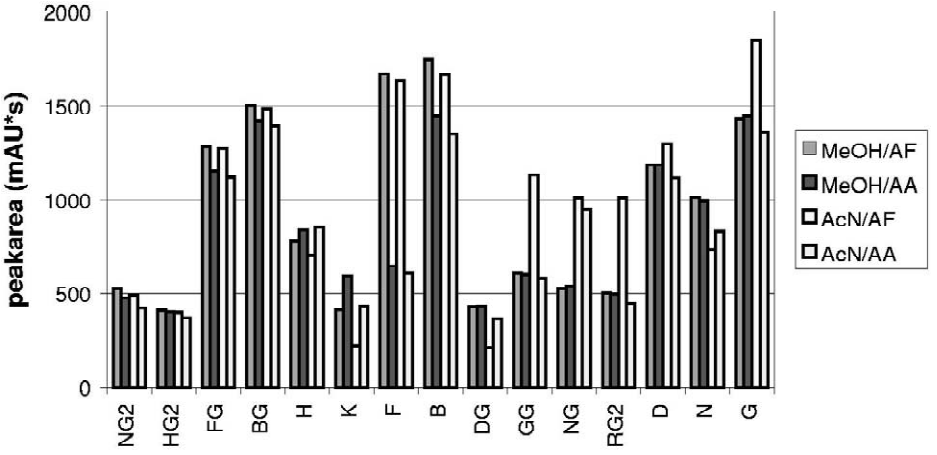


Fig. 2. Effect of LC eluent composition on the UV signal of 15 flavonoids expressed as peak areas at 250 nm (for FG, F, DG, D), 265 nm (for BG, K, B, GG, RG2, G) or 290 nm (for NG2, HG2, H, NG, N). AF and AA: pH 4.0.

RG2, G) or 290 nm (NG2, HG2, H, NG, N), depending on the absorption maximum of each compound (abbreviations are explained in Fig. 1). All flavonoids can be detected with all buffer–modifier combinations tested, but it depends on the compound which combination is best. For most flavonoids, the highest peak areas were obtained with MeOH–AF, but G, GG, NG and RG2 gave up to 50% higher responses when using ACN as a modifier. For K and DG, the responses were relatively low when using ACN–AF. The choice of the buffer, AF or AA, was found to be most important for F. To the best of our knowledge, such phenomena have not been reported and/or discussed in the literature. It has to be noted that we found that FG is not stable in pure ACN solution; a few minutes after dissolution a degradation product is formed which can be monitored in the absorption spectrum [13]. Therefore, elution with ACN may be thought also to have an effect on the degradation of other flavonoids. However, for FG in the ACN–buffer mixtures applied no significant effects were observed. Apparently, the presence of water slows down the degradation process.

In Table 3 the retention times of the flavonoids are given, with MeOH–AF or ACN–AF as LC eluent. Using AA as a buffer instead of AF has no influence on the retention times. With the MeOH-based gradient, the retention times of all compounds were longer than with ACN. With the eight-compound mixture, the differences were 1.4–3.4 min, and with the seven-compound mixture, 2.6–8.0 min. Other than that, the behavior of the analytes was essentially the same with both modifiers; the only exceptions were: a change of elution order of K and H upon exchanging MeOH for ACN and the rather rapid elution of RG2 when the latter solvent was used. Scrutiny of the literature did not reveal any paper that reports such changes of elution order upon exchanging MeOH for ACN. In all LC runs, the peaks of the glycosides were somewhat sharper when using ACN as a modifier instead of MeOH.

Table 3
Retention times (min) of (A) eight-compound mixture and (B) seven-compound mixture with two different LC gradients

	Compound	Retention time (min)	
		MeOH–AF	ACN–AF
A	NG2	7.3	5.9
	HG2	7.7	6.1
	FG	9.9	8.0
	BG	13.8	10.7
	H	14.1	12.5
	K	15.0	12.1
	F	17.8	14.3
	B	19.5	18.3
B	DG	7.9	3.7
	GG	10.7	6.5
	NG	12.3	8.9
	RG2	12.5	4.5
	D	18.3	12.1
	N	20.2	17.6
	G	21.2	17.5

The influence of the buffer pH on the UV signal was studied with the MeOH–AF gradient as an example. The pH of the AF buffer was varied between 2.8 and 4.7. Changing the pH of the buffer had hardly any effect on the UV signal intensity (not shown): for all compounds it was constant within ±2%. Calibration plots were constructed to determine the analytical performance of the LC–UV method by injecting a dilution series of the two standard mixtures containing 0.16–20 mg/l of all flavonoids (eight data points in duplicate). R^2 values were between 0.992 and 0.999 in all cases. For all flavonoids, limits of detection (LODs) ($S/N=3$) were found to be 0.01–0.16 mg/l.

One may conclude that for LC–UV, the MeOH–AF (pH 4.0) combination is to be preferred. However, in several cases ACN–AF also yields good results. Furthermore, in-line recording of the UV response was an appropriate tool to monitor the quality of the LC runs in the subsequent LC–MS studies.

3.2. LC–MS

Because our goal was to develop a method compatible with LC–UV *and* LC–MS, we did not yet discard any of the four eluent combinations, especially not because the buffer pH was expected to play an important role in LC–MS. In all cases, a UV detector was used in-line to verify the undisturbed operation of the system. Peak areas of the 15 flavonoids in the LC–MS full-scan chromatograms were compared for the four buffer–modifier combinations. Data were recorded on the quadrupole and

the ion-trap MS in both the PI and NI, and ESI and APCI modes. The results obtained on the two instruments were similar. The highest responses were obtained in the NI modes. For all flavonoids, the differences in response observed for the ionization modes were very large (up to several orders of magnitudes), much larger than the differences observed for the eluent combinations (up to 5-fold). In choosing the best ionization mode, the main criterion was the detectability of as many compounds as possible in the mixtures. We also considered a high signal in combination with a low background. For all ionization modes considered, the only exception being NI APCI, one or more flavonoids—especially the glycosides—could not be detected with any of the eluent combinations tested (data not shown) and in both positive modes the background signal was high. In both APCI modes MeOH–AF was the best eluent combination, while in the ESI modes for some flavonoids MeOH–AF was best and for others MeOH–AA. In view of the above-mentioned criteria, we focussed our attention on NI APCI. An example is shown in Fig. 3; these results were obtained on the quadrupole instrument in the full-scan NI APCI mode. All flavonoids except RG2 could be detected with all four eluent combinations (200 ng injected). The repeatability of the results was satisfactory.

As regards the differences in detectability, one example is that the glycosides have a much lower response than the aglycons. With reference to Fig. 1, one can see that if R_2=H for the isoflavones or R_3=OH for the flavones, the intensity is much higher than if R_2=Gly for the isoflavones or R_3=OGly for the flavones. These differences, which cannot be explained by the less than 2-fold differences in molar concentrations between the glycosides and corresponding aglycons, are apparently related to the nature of the phenolic OH groups. An aglycon with a free OH group is more easily dissociated into a stable anion than a glycoside. Another explanation may be that glycosides have a higher vaporization temperature so that they cannot be vaporized as efficiently as aglycones.

For most flavonoids (11 out of 15), MeOH–AF was found to give the best results, although for several compounds either MeOH–AA or ACN–AA gave similar responses. The good results obtained with AF as buffer may be explained by the stronger acidity of formic acid compared to acetic acid. Both gas-phase and liquid-phase phase acidities may play a role; also the gas phase acidity of formic acid seems to be higher than that of acetic acid [14]. The higher anion concentration of the former acid at pH 4.0 will cause the ionization efficiency to be higher also. Unexpectedly, the use of ACN–AF often led to 2–3-fold lower peak areas; apparently, the ionization efficiency is much lower.

Using the MeOH–AF gradient, the influence of

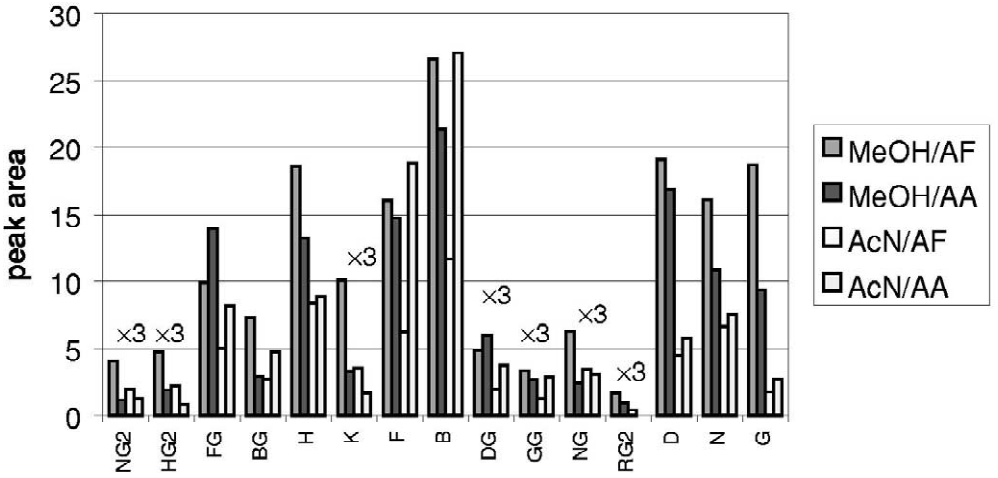


Fig. 3. Effect of LC-eluent composition on the NI APCI full-scan MS signal of 15 flavonoids. AF and AA: pH 4.0. For LC conditions, see Section 2.2.

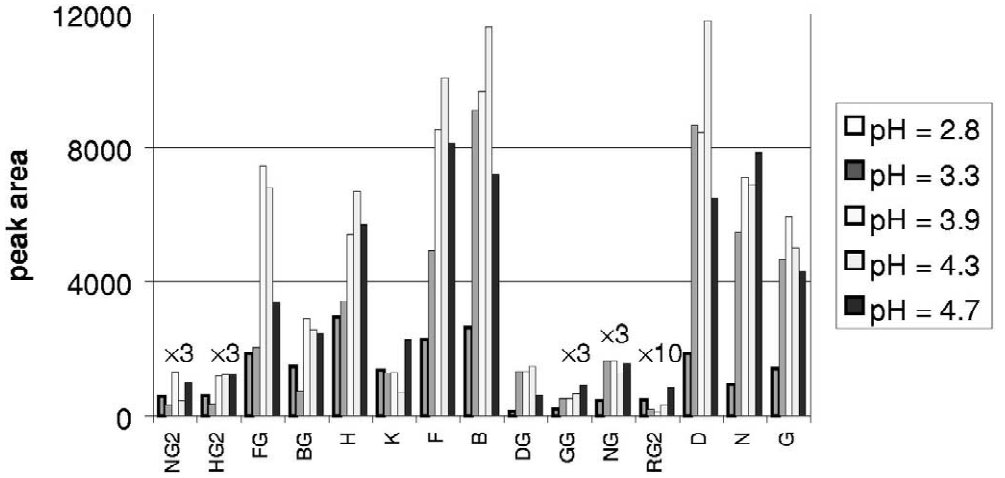


Fig. 4. Effect of AF buffer pH (MeOH used as modifier) on the extracted ion quadrupole NI APCI–MS signals of $[M-H]^-$ of 15 flavonoids. For LC conditions, see Section 2.2.

the buffer pH on the MS signal intensity of the 15 compounds was studied in the 2.8–4.7 range. Fig. 4 shows that for most compounds the extracted-ion MS signal ($[M-H]^-$) is highest for pH 3.8–4.3. Proton abstraction from phenolic OH groups to form $[M-H]^-$ anions should be more efficient at higher pH values and a similar mechanism is presumably operative in the gas phase. This trend is indeed observed but the highest pH value studied, pH 4.7, does not give the highest ionization efficiency. It is interesting to note that this phenomenon has been reported before for ion-spray (IS) and has been related to a progressively less efficient ion evaporation of anions at higher pH values [11]. In Fig. 4 extracted-ion data were used because in the full-scan MS chromatograms recorded at lower pHs, the peaks of some of the analytes could hardly be observed against the background noise. This noise is probably caused by the complexation of contaminants present in the eluent with the excess of hydrogen ions. More importantly for the present study, the relative intensities of the extracted-ion and full-scan data were found to be similar at the several pH values tested. For the rest, within the pH range tested, an optimum was reached at pH 4.0, which was used for all further work.

LODs ($S/N=3$) were determined on both instruments from full-scan chromatograms recorded using the MeOH–AF eluent combination (Table 4). On both machines, the LODs are lower in the NI than in the PI mode for both ESI and APCI, primarily because of the stronger background noise that is observed in the PI mode. The median values are in the 1–5-mg/l range. Generally speaking there is not too much difference between the LODs calculated for the two instruments irrespective of the ionization mode used, and even some of the PI-mode operations may come in useful in specific situations (also see below). Although no detailed study was made of the detectability enhancement that can be achieved via the use of extracted-ion traces, the general experience gained when constructing Fig. 4 was that a 2–3-order improvement, viz. to low-μg/l concentrations, was generally obtained.

Table 4
Ranges and median values (between parentheses) of full-scan LODs (mg/l) of 15 flavonoids in LC–MS[a]

Instrument	ESI		APCI	
	NI	PI	NI	PI
Ion trap	0.1–4 (1)	0.1–20 (5)	0.1–30 (3)	2–40 (14)
Quadrupole	0.2–30 (5)	1–50 (15)	0.2–30 (3)	0.4–30 (8)

[a] MeOH–AF eluent; for details, see text.

3.3. Ionization mode selection

As a more detailed presentation of the general observations summarized above, Fig. 5 shows the S/N ratios of all flavonoids on both instruments and for all four ionization modes. The preferred MeOH–AF (pH 4.0) gradient was used in all instances. As was already indicated above, in almost all cases, the responses are best in the NI mode, with APCI overall in first, and ESI in second place. However, some additional conclusions can be drawn. On the quadrupole MS, PI APCI also seems to be a good choice for the eight isoflavones, but not for the other flavonoids. On the ion-trap MS, however, the results obtained with PI APCI are very poor for all analytes, while PI ESI yields satisfactory results in several cases (specifically D and DG). We have no explanation for this behavior and, to the best of our knowledge, the published literature does not provide any clues either. In conclusion, a single LC–MS run using NI APCI will suffice to obtain good results for all test analytes but RG2, on either instrument. If, for increased confidence, an additional run is preferred, NI ESI can be performed.

Finally, Fig. 6 shows LC–MS traces of the eight-compound mixture with APCI (A) and the seven-

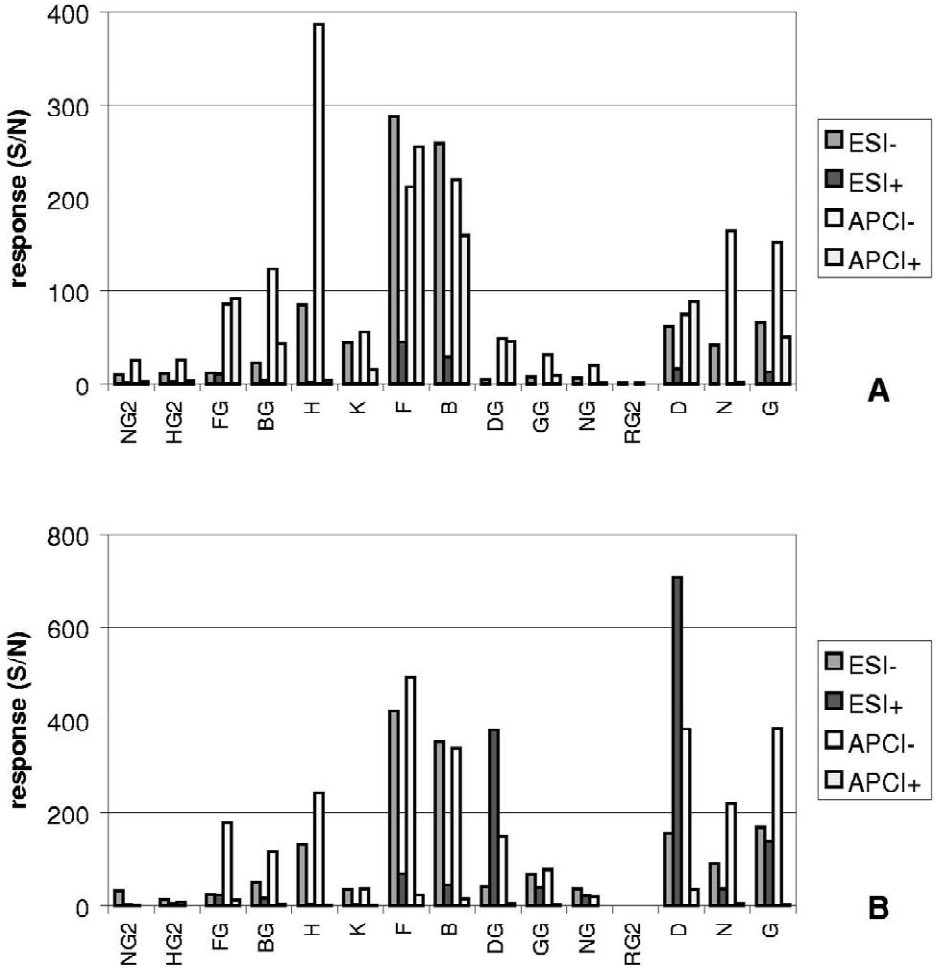


Fig. 5. Response (S/N) of 15 flavonoids with different ionization modes in full scan on (A) quadrupole and (B) ion-trap instrument. LC eluent: MeOH–AF (pH 4.0).

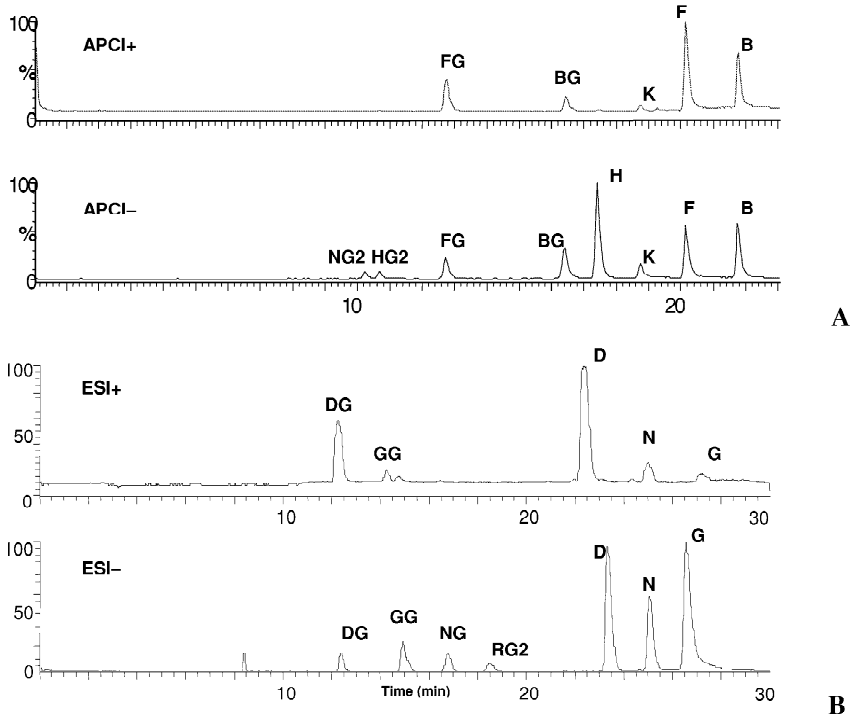


Fig. 6. Full-scan LC–MS of (A) eight-compound mixture using PI and NI APCI on triple-quadrupole instrument and (B) seven-compound mixture in PI and NI ESI on ion-trap instrument. LC eluent: MeOH–AF (pH 4.0).

compound mixture with ESI (B), in both cases using PI and NI. One observation that can now be made more easily than before is that the NI versus PI differences are much more dramatic when ESI is used. With NI, stable phenolate anions are formed. In PI, the noise level is relatively high and, under the conditions used, some peaks actually disappear in the background. A possible explanation for the low signal intensity is that the flavonoids, which do not contain nitrogen atoms, have a low basicity in the liquid phase and therefore the cation formation will be low for PI ESI.

3.4. Analyte behavior under various MS conditions

The main ions that showed up in the mass spectra of the flavonoids were: the pseudo-molecular ions, $[M–H]^-$ or $[M+H]^+$, a formic acid adduct, $[M+45]^-$, and—when the analyte was a glycoside—$[M–Gly]^-$ or $[M–2Gly]^-$, caused by loss of glycose, which agrees with literature data [1,5–7,9,10]. For the diglycosides, fragments formed by the loss of a single glycose moiety were not observed. Table 5 summarizes the results obtained for all four ionization modes, while grouping the analytes into flavonoids with two, one or no glycose moieties. Three ranges of relative abundances were used, 50–100, 10–50 and 0–10%. The three most striking conclusions that can be drawn from the table are: (i) formic acid adduct formation occurs in the NI modes only; (ii) there is more fragmentation in ESI than in APCI, but only in the PI mode; (iii) generally speaking, the mass spectra on the ion-trap and the quadrupole instruments are closely similar.

As regards formic acid adducts (for the glycosides only), in NI APCI and NI ESI the adduct formation of an available anion, formate, with a neutral flavonoid molecule successfully competes with proton abstraction from the weakly acidic flavonoids by an available base to form an $[M–H]^-$ anion. This is demonstrated by the relatively high abundance of $[M+45]^-$ ions. In PI, on the other hand, protonation of flavonoids by a suitable proton donor is a facile and a thermodynamically favorable process. Conse-

Table 5
Relative abundances of main fragments of flavonoids with zero, one or two glycose moieties in ESI and APCI mass spectra

Ionization mode	Glycose groups	M–H or M+H	M+FA	M-2Gly	M-Gly
NI APCI	2	+	–	+	
	1	–	±		+
	0	+	–		
PI APCI	2	+	–	–	
	1	±	–		+
	0	+	–		
NI ESI	2	+	±	±	
	1	±	±		±
	0	+	–		
PI ESI	2	+	–	–	
	1	±	–		±
	0	+	–		

+, 50–100%; ±, 10–50%; –, 0–10%.

quently, adduct formation to form stable $[M+47]^+$ ions is not favored.

As regards the mass spectra, there generally is little difference between the ESI and APCI mass spectra in PI, while in NI more fragments are formed in ESI. Apparently, in the latter instance more in-source fragmentation takes place. In PI, stable protonated ions are formed which do not show extensive fragmentation, while in NI less stable adduct ions are formed that are more prone to fragmentation. In addition, one should consider that different probes are used for ESI and APCI. That is, gas flows and kinetic energies are somewhat different, which can affect the fragmentation.

One somewhat unexpected observation is the difference in fragmentation for flavonoids having two or one glycose moiety: only the latter class shows appreciable aglycon formation, even though the charge is on the flavonoid part of the molecule and the bond strength between that part and the single or double glycose moiety is the same.

Thirdly, irrespective of the ionization mode selected, the mass spectra acquired on the ion-trap and the quadrupole instrument were highly similar. This close similarity allowed us to summarize the results from both instruments in a single set of data (Table 5). The only exception was that, for some flavonoids, more formic acid adducts were formed on the ion-trap MS. The most striking example is NI APCI MS of DG (daidzin; one glycose moiety). On the ion-trap instrument, the $[M–Gly]^-/[M+45]^-$ ratio was 1:1 and $[M–H]^-$ was not observed; on the quadrupole instrument, however, the $[M–Gly]^-/[M+45]^-$ ratio was 20:1, and $[M–H]^-$ had a relative abundance of 25%. We have no explanation for this exceptional behavior.

3.5. MS–MS experiments

In MS–MS studies, mass-selected precursor ions are fragmented and the product ions, which provide important structural information, are recorded. In a triple-quadrupole instrument the ions that are generated in the source are continuously transmitted to the first quadrupole for precursor-ion selection, to the octapole collision cell for collision-induced dissociation (CID) and to the third quadrupole for product-ion separation and detection. The various steps take place simultaneously but are separated in space. With an ion-trap instrument, however, these steps are separated in time, with a sequence of events taking place in the same space [15].

The MS–MS spectra of the flavonoids will be discussed for the more important NI mode, and focusing on the aglycons, because in general, apart from the loss of a glycose moiety, glycosides have the same spectra as the corresponding aglycons. One important observation, based on studying the seven aglycons, was that essentially the same results were obtained for flavones and isoflavones. Briefly, in the MS–MS spectra, $[M–H]^-$ fragments showed up in all cases and, if a methyl group is present, [M–H–

$CH_3]^-$ fragments were also observed. Furthermore, fragments formed by the loss of one or more CO and/or CO_2 groups, or by retro-Diels–Alder (RDA) fragmentation pathways were observed. For RDA cleavages two types of fragmentation are observed, which generate $^{x,y}A^-$ or $^{x,y}B^-$ ions. These refer to the fragments containing A- and B-rings, respectively; the superscripts *x* and *y* indicate the C-ring bonds that have been broken (Fig. 7). For the isoflavones, $^{0,4}B^-$ RDA fragments were mainly observed, while $^{1,3}A^-$ fragments were more prominent in the case of the flavones. As an illustration, the MS–MS spectra of biochanin A are shown in Fig. 8. In Fig. 8A fragmentation on the ion-trap instrument by CID at 50% activation energy takes place in four separate steps in time (MS to MS^4). In contrast, the tandem MS spectrum at 20-eV collision energy on the triple-quadrupole instrument (Fig. 8B) is the result of a one-step process in which several fragment ions were formed.

The main conclusion of the study was that—for all analytes tested—the sets of MS–MS spectra recorded on the ion-trap and triple-quadrupole instruments showed that in general the same fragmentation reactions are observed. The advantage of the ion-trap is that it offers the additional possibility to ascertain precursor→product ion relationships.

3.6. Comparison with literature data

Recently, there have been many publications that deal with the determination of flavonoids in various types of samples. A brief comparison with literature values is therefore indicated. Most authors listed in Table 1 above used only one set of conditions, i.e. one eluent and usually only one ionization mode; moreover, in most papers, there is no information on LODs. Only six out of the 12 papers in the table used both the PI and NI modes and only three compared different ionization techniques.

From among the authors using only one ionization mode, two groups rationalized their choice, in both cases NI, by stating that although in most studies PI modes are used, NI APCI is excellent for flavonoid analysis, both regarding specific and structural in-

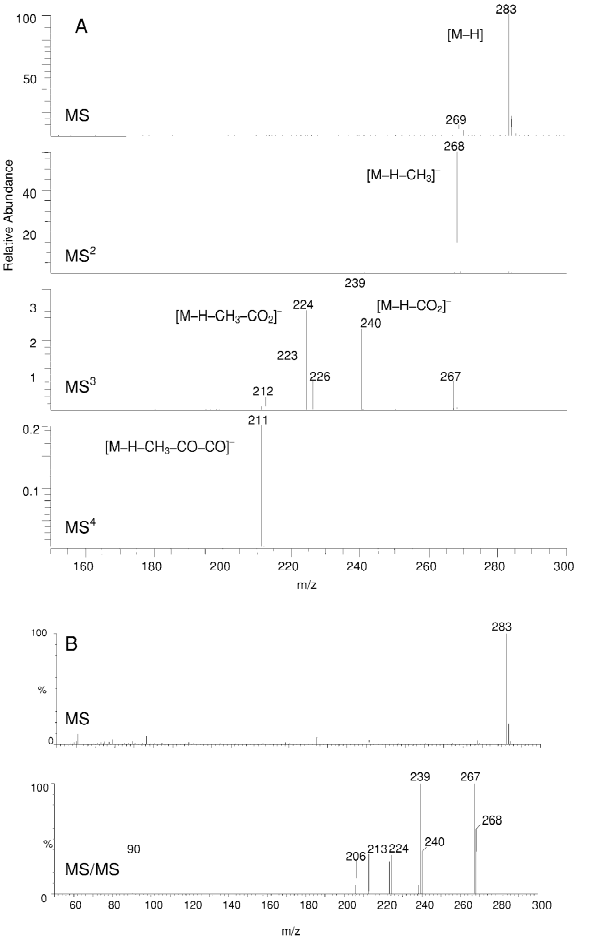


Fig. 8. MS–MS spectra of biochanin A on (A) ion-trap and (B) triple quadrupole instruments. For LC and MS conditions, see text.

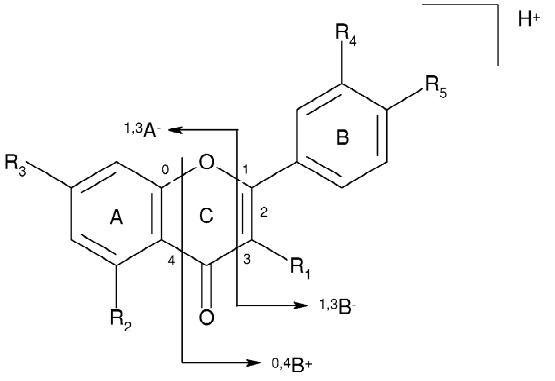


Fig. 7. Nomenclature for the different retrocyclization cleavages observed (adapted from Ref. [16]). For explanation see text.

formation [1], while NI ESI appeared to be more sensitive and selective than PI ESI for the analysis of plant material [9]. These statements agree with our findings.

Some of the authors who used various ionization modes stated that for identification purposes spectra recorded in the PI mode were more easy to interpret because they showed more characteristic fragments than spectra recorded in NI; however, the latter type provided better detectability [2,5]. Some papers focus attention on the interpretation of the mass spectra of the flavonoids [6,7] and the various ionization modes are not discussed in much detail.

Rauha et al. [11] compared the use of different LC eluent compositions in APCI, IS, and also APPI, but the latter technique is outside the scope of the present study. Using the sum of the areas of the most abundant ions in the mass spectra they found, in accordance with our results, that for PI APCI the best sensitivity is achieved with formic acid as eluent. They also noted that PI gives a much stronger background noise than NI. Seemingly in contrast with our results, these authors reported that in NI APCI the best sensitivity is obtained with AA. However, pH adjustment to 4.0 (also our optimum) was done by adding formic acid, which renders conditions closely similar to ours. In positive IS (which is comparable to our ESI mode of operation) Rauha et al. found that in total ion current (TIC) the best sensitivity was obtained with formic acid as an eluent while a decrease of sensitivity was observed when the formic acid concentration was lowered. The use of ammonia improved the system robustness and made it less dependent on pH; however, the presence of ammonium ions in AA decreased the sensitivity. In negative IS in TIC the best sensitivity was obtained with an eluent containing formic acid at low pH. For AA, they found a decreased sensitivity, which might indicate ion suppression by AA or neutralization of negative charges by the ammonium anions. In line with this interpretation it was observed that increase of the concentration from 10 to 20 m*M* reduced the sensitivity for all flavonoids tested. The results of Rauha et al. cannot be straightforwardly compared with ours, since in all eluent compositions considered above ammonium ions were present to obtain an adequate LC-separation. Although the authors used other test flavonoids, the LODs were similar to ours, viz. 1–4 mg/l in NI APCI and 2–37 mg/l in PI APCI.

Several papers included in Table 1 provide information on detectability. LODs based on UV data of flavonoids in onion and soybean were 6–42 pmol injected [4], which corresponds with analyte concentrations of 0.08–0.63 mg/l and agrees with our findings. More importantly, one study on MS detection reports LODs in the low-μg range when using an ACN–trifluoroacetic acid gradient and full-scan PI ESI–MS detection [10]. These are fairly high values. This can probably be explained by the use of conditions, which, according to our present experiences, certainly are not optimal. Much better results were reported in another paper on the determination of flavonoids in urine using MeOH–ACN–formic acid as eluent, and NI APCI-mode detection. With selected ion monitoring, which, of course, offers a considerable gain in selectivity as well as sensitivity, LODs were 0.25–2.5 ng/ml [8]. These values are of the same order-of-magnitude as the extracted-ion-based data reported in Section 3.2 above.

4. Conclusions

The responses of 15 flavonoids were compared in LC–UV and LC–MS using four different eluent compositions, while varying the pH, and four different API modes. In LC–UV, all test compounds could be detected with approximately the same sensitivity; responses varied by no more than a factor of three. For LC–MS, however, much larger differences were observed; responses varied up to two orders of magnitude both between analytes and between various sets of experimental conditions. This implies that one should be cautious when interpreting data on the screening of real-life samples. In general, the eluent combination methanol–ammonium formate buffer at pH 4.0 gave the highest responses. MS responses were best in the NI modes, with APCI overall in first, and ESI in second place. It is interesting to add that the results obtained with NI APCI and NI ESI were closely similar for all aglycons. This has some practical value because, in many studies, the glycosides present in a sample are hydrolyzed prior to analysis.

When the performance of both instruments was compared, it was found that for each of the four ionization modes, the responses were essentially the same on both machines. Three main ions are formed: $[M-H]^-$ or $[M+H]^+$, $[M+45]^-$ and $[M-2Gly]^-$ or $[M-Gly]^-$, with formic acid adduct formation occurring in NI modes only and fragmentation being somewhat more prominent in the ESI mode. More importantly, closely similar mass spectra were generated by both MS instruments. The fragmentation reactions in MS–MS were also found to be generally the same on the ion-trap and the triple-quadrupole. Since analyte detectability on the ion-trap instrument is, today, better than it used to be some years ago [15] and is now fully comparable to that found for the quadrupole, the additional possibility to ascertain the precursor→product ion relationships seems to favor the use of the former instrument.

Finally, in the literature, not too much attention has been devoted to a systematic comparison of different ionization modes and/or LC eluents for flavonoids. Still, a close scrutiny of the published data reveals that most of these agree with our preferred approach, the use of MeOH–AF (pH 4.0) with NI APCI mass spectrometry. Currently, the method is being used—on the ion-trap instrument—to detect and identify flavonoids, both target compounds and unknowns, in wetland plants.

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