Review

Chromatographic, capillary electrophoretic and capillary electrochromatographic techniques in the analysis of flavonoids

I. Molnár-Perl*, Zs. Füzfai

Institute of Inorganic and Analytical Chemistry, L. Eötvös University, Budapest 112, H-1518, P.O. Box 32, Hungary

Abstract

An overview is presented of chromatographic methods currently in use to determine flavonoids, including free aglycones, their corresponding glycosides, one by one, and, in the presence of each other. As a basis of selection, the following approaches can be distinguished: critical evaluation of the preliminary steps (extraction/isolation and hydrolysis) as well as the separation, identification and quantitation of constituents both on the basic research level and/or subsequently to various work up procedures. Chromatographic techniques were discussed after extraction/isolation of various flavonoids from several natural matrices. Papers were classified and compared from analytical point of view, primarily on the chromatographic, secondly on the detection techniques applied.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Reviews; Flavonoid's analysis; Chromatography; Aglycone; Flavonoid glycoside

Contents

1. Introduction

In spite of the relevancy of flavonoids and the tremendous amount of papers dealing with the identification and quantitation of different flavonoid species in various matrices, according to author's knowledge, there is no recent review article associated with their chromatographic analysis. This fact is not a chance, it is thoroughly associated with the complexity of the task: with the very special chemical, physical and structural properties of flavonoids, with the huge number of papers containing several contradictions. All these characteristics deter analytical chemists from dealing with the topic.

The chemical structure of their main representatives, belonging to various groups of compounds, such as anthocyanidins, catechins, flavones, flavonols, flavanones and isoflavones, are depicted in Fig. 1. However, due to the intrinsic features of flavonoids:

 (i) ready to transformation/oxidation/reduction processes, intra and intermolecular rearrangements,
(ii) being different in number and positions of their hydroxyl groups, and
(iii) being linked to several saccharides of various structures and degree of polymerization, the versions of a single flavonoid might be huge. Taking into account the diversities of the possible species, it turned out that theoretically more than 2×10^6 flavonoids can exist and for the time being more than 2×10^3 species have been already identified [1]. For instance, quercetin alone does have more than 179 glycosides [2,3].

The relevancy of these group of organics (being regarded as versatile, beneficial impact furnishing natural compounds) is associated with their invaluable physiological/biological and practical characteristics [4,5]. It has been confirmed that flavonoids of polyphenolic structure and of antioxidant characteristics (identified in almost all plants, vegetables and fruits, mainly in the form of their β-glycosides [5]), in living organism become absorbed very fast [4]. According to epidemiological studies, flavonoid containing vegetables and fruits demonstrate a protective effect against cancer, stroke and coronary heart disease related to their antioxidant properties [5]. Antimutagenetic activity of flavonoids (sourced from the heartwood of Rhus verniciflua) was proven on bromobenzene treated rats [6].

Thus, on the basis of the usefulness and importance of these types of natural compounds, it seemed to be a need to summarize recent chromatographic procedures.

No doubt about it, the complete analysis of a flavonoid-including its detailed structure, configuration of its ring positions, numbers of double bonds, free and substituted hydroxyl groups and additional substituents of the rings-needs a cooperation of analytical chemists, as well as the availability of advanced separation and identification techniques, at once [chromatography, nuclear magnetic resonance (NMR) and mass spectrometry (MS), etc.]. Certainly, the best solution of this task would be a HPLC–MS–NMR apparatus that can fulfill all these requirements, simultaneously [7]. Unfortunately, only a few laboratories are equipped with this relatively new system. However, simpler approaches, such as high-performance liquid chromatogra-
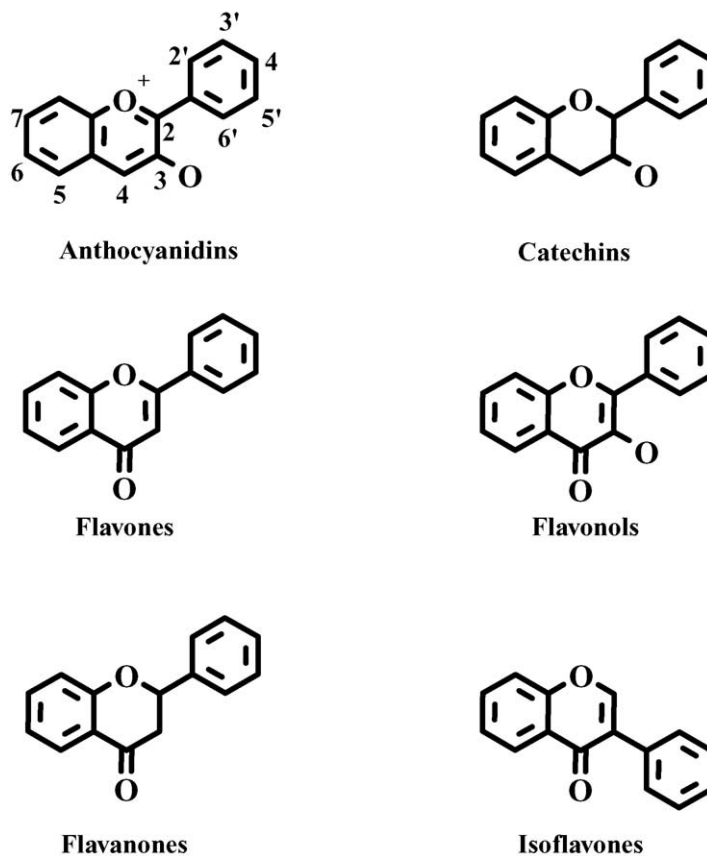
Fig. 1. Chemical structure of the main types of flavonoids.

phy (HPLC), capillary electrophoresis (CE), capillary electrochromatography (CEC) or gas chromatography (GC) can provide very useful information, in terms of identification and quantitation, equally, furnishing excellent resolution and selective retention times. Chromatography, completed with different, in overwhelming cases, coupled detection systems [HPLC: UV–fluorescence (FL), photodiodearray (DAD)–FL, UV(DAD)–MS; GC: GC–flame ionization detection (FID), GC–MS, GC–MS–MS] enhance the certainty of analytical results, exponentially. Thus, it is understandable that special, well limited topics of hyphenated techniques, relating to flavonoids, were reviewed [8–10]: such as mass spectrometry for identification/structural studies of flavonoid glycosides [8], hyphenated techniques used in the phytochemical investigation of legume-flavonoids [9] and LC–MS systems in the analysis of food flavonoids [10].

The aim of this compilation is to give an overview and evaluation on complete chromatographic analysis of flavonoids (including extraction/isolation procedures, separation, identification and quantification by different chromatographic techniques), focusing to proposals published mainly in the late nineties and in 2000–2004.

Classification's principle of papers was relied primarily on the chromatographic, secondly on the detection techniques applied. Selections related to the main, from analytical point of view the final results definitely determining steps of analyses.

2. Extraction/isolation procedures

This starting step of the analytical procedure is regarded an obligatory part of process, independently on the subsequently applied chromatographic and detection methodologies such as HPLC [7,11–89], CE/CEC [90–98] or GC [104–128], with the exceptions of model studies [7,67,69–71,75,79,93,98,103,104,107,112,115] and of a single, early proposal [114] relating to the simultaneous extraction/derivatization process for the analysis of rutin, in a dried herb sample, in the presence of the matrix.

Selected isolation/extraction procedures of flavonoids are compiled according to the matrix they are to be separated from (Table 1 [16,17,22,28,31,37,52,58,60,61,62, 64,73,77,80,125,130–132]).

The two, main types of isolation processes, classical extraction with aqueous methanol and solid-phase extraction (SPE), proved to be the most commonly used ones (Table 1, Sections 2.1–2.3).

On the basis of an overview of more than five hundred papers (selected for detailed review 133), it became clear that the traditional approach to find optimum condition, by varying

Table 1
Isolation/extraction conditions of flavonoids from various matrices

The matrix	Flavonoid(s) to be extracted	Extraction conditions		Limit of detection	Recovery, R.S.D. (%)	Identifica-tion/quantitat ion	Reference, year
		Type	Procedure				
Biological matrices							
Human plasma	Naringin, naringenin	Sep-Pak t C_{18} filtration	1 mL plasma + 2 mL H_2O; SPE (pw: 5 mL Met + 5mL H_2O); El: 2.5 mL H_2O + 5 ml 80% Met; Fil: HLC disc	0.2 ng	2.6–6.0	HPLC–UV	[16] 1996
Human urine	Naringin, naringenin	Sep-Pak Accell QMA, filtration	1 mL urine + 2 mL H_2O; SPE (pw: 15 mL Met + 15 mL H_2O); El: 10/7 mL Met cont: 10/4 mM HCOOH (narin-genin/naringin); Fil: HLC disc	1–5 ng	1.8–9.0	HPLC–UV	[17] 1997
Rat plasma	Apigenin	Vortex mixing, centrifugation	0.5 mL plasma + 0.8 mL Me: vort mix (60 s), centr. (2200 g, 4 °C, 15 min) evap	∼6 ng	0.4-5.6	HPLC–UV	[22] 2000
Human urine	Quercetin, kaempferol from tablet of ginkgo biloba extract	Rotational extraction	4 mL urine + 1 mL 25% HCl (hydr: 30 min, 80 °C) + 5 mL ether extraction (5 min) centr 10 min, evap to dryness	4–4.4 ng	<9.7	HPLC–UV	[28] 2003
Human urine, plasma	Theaflavins: epigallocaetchine, epicatohine, epigal-locatechine gallate, epicatechine gallate, theaflavine, theaflavine-3-gallate, theaflavine-3′-gallate, theaflavine-3,3′-digallate	Vortex mixing, extraction, centrifu-gation	1 mL plasma/urine + ascorbic acid (pH 5); hydr + 100 μl β-glucuronidase/sulfatase, 37 °C, 1 h; 500 μL aceton vort mix; 2 mL × 2 mL ethyl acetate vort mix (5 min, 2500 × g), evap (37 °C, N_2)	120 pg	4.4–17.1	HPLC–MS (ESI)	[72] 2001
Human urine	Quercetin, kaempferol from tablet of ginkgo biloba extract	SPE: isolute (100 mg/mL) ENV^+ cartridge	1 mL centr. urine + 0.5 mL 3 M HCl (hydr: 1 h, 80 °C) + 1 mL 1 M Phos. buffer (pH 6.8); SPE (pw: 1 mL Met + 0.8 mL H_2O); El: 3× 0.7% Met + 0.01 M TFA cont H_2O, then 2 mL × 0.5 mL ACN/H_2O: 8/2, evap; deriv.	10 pg	<9.4	GC–MS	D.O. Watson, J. Chromatogr. B, 1999
Fruit juices							
Orange, grapefruit	Hesperidin, naringin, nairutin	Vortex mixing, extraction, centrifu-gation, filtration	1 g grapefruit juice + 3 mL × 1 mL Met, vort mix (1 min), centr (25 000 × g, 15 min), 3 mL unified supernatant + 2 mL H_2O centr, Fil: 1 μm glass fiber then 0.2 μm Anotop: 1 g orange juice + 4 mL Met (55 °C), etc. (Note, extraction optimization study: solvent, temperature, number of extr steps)	–	–	HPLC–DAD	[31] 1995
Orange, grapefruit	Flavanone glycosides: naimtin, naringin, hesperidin, neohesperidin, didymin, poncirin; polymethoxylated flavones: sinensetin, hexa- and heptamethoxyflavon, nobiletin, scutallerain, tangeretin	Centrifugation, filtration	25 mL fruit juice + 20 mL DMF (water bath: 10 min, 90 °C) cooling adjusting to 50.0 mL, centr, Fil: Aerodise filters (5 and 0.45 μm)	50 ng–1 μg	<6.4	HPLC–DAD	[37] 1998
Orange	Flavanone glycosides: eriocitrin, neoe-riocitrin, naiiutin, naringin, lesperidin, neohesperidin; flavonols: quercetin, kaempferot, jalangro; flavones: api-genin, chrysin	Extraction, centrifiigation, filtration	Extraction + hydrolysis: 3.5 g juice + 4 mL Met + 1 mL 12 M HCl + 12 mg BHT: reflux (90 °C, 1 h), cooling, +Met to 10 mL; sonication (5 min); Fil: PTFE (0.45 μm) (Note, hydrolysis study: HCl concentration, time)	ED/UV 20–50/40–80 ng	ED/UV, <10/<4	HPLC–DAD; HPLC–ED	[50] 2000
Fresh fruits, vegetables, herbs							
Willow herbs, 13 Epilobium species	Flavanone glycosides (3-O-glycosides of kaempferol): uercitrin, guajaverin, hyperosid, isoquerettrin, tnyricitrin, isomyricitrin	Isolation, fractionation	1.1 kg dried aerial plant parts extracted successively with 1. CH_2Cl_2, 2. met, 3. n-But; yield: 23 g, fractionated by gel filtration (Sephadex LH-20, Met); Frac-tions purified by micropreparative HPLC	–	–	HPLC–UV HPLC–MS (TSP), ^{1}H NMR, ^{13}C NMR	[57] 1995

Sour orange	Flavanones: isonaringin, naringenin, naringin, hespereritin, lesperidin, neohesperidin; flavons: tageritin, no-biletin; avonols: auranetin, quercetin; synephrine	Extraction, filtration	1.2 g dried, ground fruit + 50 mL 80% Et (90 °C, 2 h), filtered dried at vac-cum: 120 mg extract dissolved in 50 mL Met filterd (nylon acro-disk, 13 mm, 0.45 nm)	–	–	HPLC–UV–MS(ES)	[59] 1997
Leaf of Cistus ladanifer L.	Apigenin, 4′-methyi-apigenin, 7-methyt-apigenin, 7,4′-dime-nethyl-apigenin, 3-methyl-kaempferol, 3,4-dimethyl-kaem-ferol, 3,7-dimethyl-kaempferol, 3,7,4′-trimethyl-kaempferol	Extraction (isolation, fractionation)	3–4 leafs (0.25–030 g) extracted by 5 mL × 2 mL CHCl₃, dried, redissolved in Met (1 g extr/50 mL Met), cooled at −20 °C overnight: waxes removed; fractionation: Sephadex LH-20 column (12.5 g, 25 cm long, 1.5 cm Ø)	–	–	HPLC–UV–PB-MS(ES)	[60] 1998
Apple, pear, orange tomato, onion, tea broccoli, etc.	Nyricetin, quercetin, naringmin, lute-olin, hespere-n, kaempferol, apigenin, eriodictyol, phloretin. sorhamnetin	Extraction, filtration	Food's edible part (peelseparately) chopped, lyophilised, kept at −18 °C. Extr + hydr: 5 g freeze-dried, pulverised sample + 40 mL 62.5% aqueous Met cont BHA (2 g/L) + 10 M 6 M HCl (steam bath, 90 °C, 2 h reftux), diluted to 100 mL with Met, sonicated (5 min); Extr without hydr 5 g freeze-dtied, pul-verized sample + 20 mL 62.5% aqneous Met cont BHA (2 g/L); 2 mL sedimented extract + 2 mL H₂O (pH 2.5) fil	1 ng	<13	HPLC–UV–MS (APcI)	[61] 1998
Three cultivaed, seven wild berries	Flavonols: kaempferol, quercetin, myricetin	Extraction, centrifugation	Extraction: 5 g homogenised berry + 25 mL H₂O + 25 mL Met was shaken (N₂, 21 °C, 2 h) and centritbged. 20 mL sttpematant evap, dissolved in 1.5 mL Met + fil (0.45 µm), Extr + hydr: 5 g homogenized berry + H₂O upto 15 mL + 25 mL Met (cont, 2 g/L TBHQ) + 10 mL 6 M HCl: reflux 85 °C, 2 h), 20 mL filtrate evaporated, dissolved in 1 5mL Met, fil (0.45 µm)	40–200 ng	–	HPLC–DAD–MS (ESI): flavonoids GC–MS: sugars	[63] 1998
Soybean pod	Flavon agrycons: apigenin, 7,4′-dihydroxyfiavone, luteolin; flavon gly-osides: apigenin/luteoIin-7-O-β-D-glucosides, 7,4′-dihydroxyuavone-7-O-β-D-glucoside, apigenin-7-β-D-glucoside-6″-O-malonate	Extraction, centrifugation	0.3–0.6 g soybean pod homogenized with 1.5 mL 80% Et (50 °C,1 h); Cooled, centr (14 000 × g, 10 min): 100 µL su-pernatant analyzed	–	–	HPLC–DAD–(APCI)	[78] 2003
Leaf of Sorocea bomplmdii	Quercetin/kaempferol-diglycosides, kaempferol-tri-glycoside	Extraction, fractionation	88 g dried (40 °C, pulverized Ieves + 1 L H₂O (boiling, 1h); cooled, fil/frac (Am-berlite XAD-2 column, 25 cm × 3.5 cm i.d., dow rate, 1mL/rnin). El: (1) 1 L H₂O, (2) 200 mL Met. Met fraction vac-uum dried. 10 mg methnolic extract dis-solved in 1 mL Met; sonication: 15 min, fil: 0.45 µm	–	–	HPLC–NMR	[81] 2002
Brined olive drupes	Luteonin, apigenin, hydroxytyrosol, phenols, phenolic acids	Extraction, micro-preparative HPLC	100 g dehulled, freezedried pericarp Soxhlet extr 2×, with pentane (3 h) to remove fat, dried ericarp extr 3×, with Met, evap (vacuo 35 °C), residue + 50 mL ACN extr 3× with hex-ane ACN solution dried (35 °C); residue suspended in 5 mL Met; microprepara-tive HPLC	–	–	GC–MS(EI) HPLC–MS(ESI)	R.W. Oven, 2003

h: human; pw: prewashed/conditioned; Fil: filtered through; H₂O: distilled water; Met: methanol; Et: ethanol; El: elution with; vort mix: vortex mixing; centr: centrifugation; DMF: dimethylformamid; BHT: butylated hydroxytoluene, antioxidant; –: no data available; TBHQ: *tert*-butylhydroxyquinone (antioxidant).

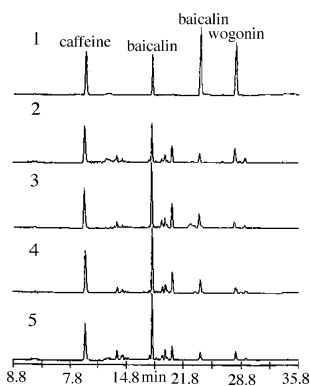I. Molnár-Perl, Zs. Füzfai / J. Chromatogr. A 1073 (2005) 201–227

Fig. 2. HPLC chromatograms of a mixture of authentic flavonoids (baicalin, baicalein, wogonin and caffeine as internal standard) obtained without extraction (1) and with different type of extraction procedures from Scutellariae radix (2–5); (2) SFE extract from supercritical CO_2 with methanol–water (70:30); (3) SFE from supercritical CO_2 with methanol; (4) extract from ultrasonic shaking with methanol–water (70:30); (5) extract from ultrasonic shaking with methanol. (With permission from [109].)

parameters one by one, remains an illusion, only: It means, composition of extracting agent to the analyte, time, temperature etc., that could have been accepted in general, in agreement also with earlier experiences [132], were not found. Optimum extraction conditions are dependent on the compound(s) and on the matrix to be isolated from. The comparison of the classical and supercritical fluid extraction (SFE) procedures based on the data of two papers [109,129] reveals contradictory evaluation (Section 2.4 Figs. 2 and 3). The pressurized liquid extraction (PLE) of the catechin/isocatechin couple was studied recently [130] (Section 2.5).

2.1. Isolation of flavonoids from biological fluids

Data in Table 1 give an overview on the amounts of samples to be extracted, on the steps of isolation processes and on the analytical characteristics of the methods. Out of 16 examples, detection limit was given in 10 cases [16,17,22,28,37,52,62,64,72,125]; reproducibility, characterized by the R.S.D. percentages is given in nine papers [16,17,22,28,37,52,62,72,125] only.

Extraction of flavonoids from biological matrices is one of the fastest and the less time consuming task (Table 1 [16,17,22,28,73,125]). In addition, due to the simple manipulation of relatively small amount of samples to be extracted, analytical characteristics, such as the limit of detection (Table 1: 10 pg–6 ng) and the relative standard deviation percentages of recoveries (R.S.D., 0.4–9.7%) proved to be attractive.

In a recent study [131], different sample preparation of wine phenolic compounds (six phenolic acids, five flavonoids, *trans*-resveratrol and tyrosol) from human plasma were compared. The recovery values of sample treatments (SPE, extraction with methanol, deproteinization, inhibition of enzymatic plasma activity) were compared. Aiming to quantitate the whole set of compounds, in this special case,
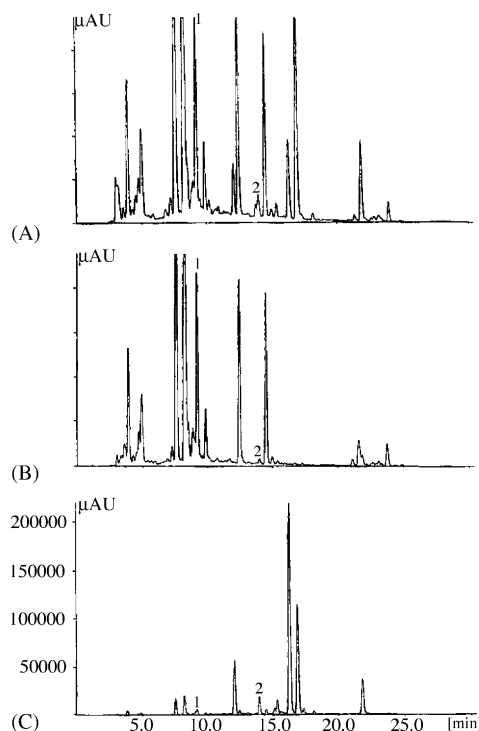
Fig. 3. HPLC chromatograms of chamomile extract obtained by Soxhlet extraction (A: 1 g, ethanol, 6 h), by maceration (B: 1 g, ethanol, 3 days) and by SFE (C: 200 atm; 40 °C; flow rate, 1 L min^{-1} gaseous CO_2; 1 atm = 101 325 Pa). Peaks: 1, apigenin-7glucoside; 2, apigenin. (With permission from [129].)

the most suitable approach was to inhibit enzymatic activity and then deproteinize with acidified ethanol.

2.2. Isolation of flavonoids from fruit juices

Juices (Table 1 [31,37,52]), primarily the fruit particles and the relatively low flavonoid containing ones, require more conscious preparations of extractions. Accordingly, higher detection limits (Table 1: 20–1000 ng) and poorer reproducibility percentages (Table 1: 4–10 R.S.D.) have been reported.

2.3. Isolation of flavonoids from fruits, vegetables, herbs, etc.

Extraction/isolation of flavonoids from solid samples (Table 1 [58,60–62,64,77,80]) are the most tedious/time consuming procedures, in particular in those cases when the final aim is to separate a single flavonoid, in pure form and in considerable amount, for 'off-line' identification purposes. Unfortunately, in these cases no, or limited attention was paid on quantitative aspects. Out of the selected eight cases only in two were provided data for detection limit (Table 1: 1 ng, 20–40 ng) and only in a single one for reproducibility (R.S.D. < 13%).

2.4. Comparison of the efficacy of supercritical fluid extraction with classical methods

The importance and utility of this, in many areas of the active component extraction of natural matrices preferred technique, must be evaluated separately depending on the flavonoid's composition [109,129] (Figs. 2 and 3). In the case of the non-polar flavonoid aglycones isolation by SFE, in comparison to classical techniques, provided identical [109] or somewhat better yields [129]. However, in the case of water-soluble glycosides the use of SFE furnished considerable different yields [109,129]. In the case of Scutellariae radix (Fig. 2) [109], comparing classical ultrasound extraction with SFE, its free aglycone (baicalein) and its corresponding flavonoid glycoside (baicalin), both were obtained in higher yield with SFE.

However, comparing the SFE recoveries of apigenin-7-glucoside from chamomile to the simple maceration and to the Soxhlet extractions (Fig. 3), in order of listing, were only 14.6% and 19.5%, respectively [129].

Aqueous methanol as an extracting solvent, in general, proved to be a useful compromise that ensures the extraction of both aglycones and flavonoid glycosides, depending on time, temperature, etc.

2.5. Pressurized liquid extraction

This technique was proposed for the isolation of catechin and epicatechin from tealeaves and from grape seeds [130]. Three extraction systems (magnetic stirring, ultrasound-assisted extraction, PLE), were compared using, four different solvents for all three systems, the same ones (water, methanol, ethanol, ethyl acetate). In comparison to any other conditions, PLE, applying methanol as solvent, provided the highest recovery with excellent reproducibility (R.S.D. < 3%).

3. Hydrolysis of the extracts

Depending on the type and on the aim of analyses a number of possibilities can be distinguished.

(i) Chromatographic separation of the flavonoid content of extract, without hydrolysis, expecting to identify and quantify free and sugar moiet(y/ies) containing flavonoids, in the presence of each other. In this term mainly HPLC techniques were used.

(ii) Separation of flavonoids from a hydrolyzed extract, in order to decrease the number of compounds to be determined, resulting in better resolution and improved characterization of the flavonoid constituents (Table 2 [30,33,35,42,43,52,59,62,64,65,83,112,124,125,127].

(iii) Separation and quantitation of both the sugar and the flavonoid content of the hydrolyzed extract: either by different methods (HPLC: flavonoids; GC: sugars), or sugars and flavonoid aglycones, simultaneously (GC–MS) [133]. (Note: Advantages of hydrolysis methodologies will be discussed also with selected examples.)

Table 2
Hydrolysis conditions of flavonoid-glycosides

Matrix	Aglycone(s) to be hydrolysed	Antioxidant	Condition: concentration relating to the final volume	Reference
Dried herb	Quercetin	–	Water–methanol (2.5:1), 2 M HCl, 1 h, reflux	[30]
Fresh fruit	Quercetin, kaempferol	–	Water–methanol (1:1), 1 M HCl, 1 h	[33]
Onion	Quercetin,	SDDC, 20 mM	Water–methanol (1:1), 1.2 M HCl, 90 °C, 2 h	[35]
Celery	Apigenin, luteolin	SDDC, 20 mM	Water–methanol (1:1), 2 M HCl, 90 °C, 4 h	[35]
Tea leaves	Myricetin, quercetin, kaempferol	–	6 M HCl, 2 h	[42]
Cranberry	Catechin, myricetin, quercetin,	80 mg AscA/50 mL	3 M HCl, 35 °C, 16 h (N_2, stirring)	[43]
Orange juice	Quercetin	–	1.5 M HCl, 1 h, model study	[52]
Dried herb	Genistin, isoquercetin, ononin, daidzein	–	70% ethanol–2 M HCl (1:2), reflux, 3 h	[59]
Dried apple, pear	Nine aglycones	80 mg BHT	Water–methanol (1:1), 1.2 M HCl, 90 °C, reflux, stb, 2 h, 4 h (red wine)	[62]
Berries	Myricetin, quercetin	TBHQU	Water–methanol (1:1) 1.2 M HCl, 2 h, 85 °C, reflux	[64]
Beer, wine	Naringenin, prenylnaringenin, etc.	–	Methanol–2 M HCl (1:1), 2 h, reflux	[65]
Tart cherry	Cyanidin	–	3 M HCl, 1 h, 100 °C	[83]
Dried herb	Ten aglycones	–	2 M HCl, 1 h, 100 °C	[124]
Human urine	Quercetin	–	1 M HCl, 1 h, 80 °C	[125]
Phytopharmaceu-tical	Bilobalides A, B, C, kaempferol, quercetin, isorhamnetin	–	1 M HCl in 20% Met, 1 h, 85 °C	[127]

h: human; pw: prewashed/conditioned; Fil: filtered through; H2O: distilled water; Met: methanol; Et: ethanol; El: elution with; vort mix: vortex mixing; centr: centrifugation; DMF: dimethylformamid; BHT: butylated hydroxytoluene, antioxidant; –: no data available; TBHQ: *tert*-butylhydroxyquinone (antioxidant); SDDC: sodium diethyl dithiocarbamate; AscA: ascorbic acid, stb: steam bath.

Evaluating various hydrolysis conditions (Table 2), even in the case of the same matrix, aiming to release the same aglycone(s) reflect the difficulty and importance of this preparation step. Under the hydrolysis process, optimum compromise is to be found to minimize degradation reactions of glycosides and to achieve complete release of aglycones. For this purpose, a central composite experimental design [52] was described to get optimum quercetin yield from the hydrolysis of rutin, present in orange juice. Applying a multiple regression analysis of data set (based on chromatographic analysis obtained under various hydrolysis conditions), it was possible to obtain a mathematical model that took linear, quadratic and cross-product terms into account [52]. According to this mathematical approach optimum condition for the hydrolysis of rutin, in orange juice, proved to be a HCl concentration of 1.5 M and a hydrolysis time of 1.5 h.

Numerous paper have been cited in an earlier exhaustive study [132] relating to the systematically tested hydrolysis conditions for flavonol glucuronides, flavonol glucosides and flavone glucosides, for six food samples. Optimum acid concentration and hydrolysis time, expressed in HCl concentration (M)/h, were reported on the quercetin, kaempferol, myricetin, luteolin and apigenin yields from cranberry, onion, leek, lettuce, endive and celery, in order of listing, 1.2 M/0.5 h, 1.2 M/2 h, 1.6 M/4 h, 2.0 M/2 h, 1.6 M/4 h and 2.0 M/4 h, respectively. These data proved to be different, indicating the fact that unified optimum conditions could not be suggested.

4. Separation of flavonoids by high-performance liquid chromatography, without derivatization [11–89]

In general, separations are followed mainly on C_{18} RP columns, differences in identification and quantitation characteristics of procedures are associated with the detection system and will be detailed according to the detection method applied.

4.1. HPLC–ultraviolet detection [11–29]

HPLC–UV, this most simple system is still common in biochemical and food analysis oriented laboratories: from human plasma [14–16], rhoifolin and daidzin [14], quercetin [15], naringin and naringenin [16], from human urine [17,28], naringin, naringenin, [17], quercetin and kaempferol [28], from rat blood [21], quercetin and catechins, from rat plasma [22], apigenin were isolated and measured. Anthocyanins of cherries [11,12,27], red grapes [18], and apples [23,24,26], quercetin and kaempferol from vegetables [20] as well as rutin and esculin from plant materials and drugs [13], hesperidin and naringin from citrus leafs [25], naringin, gossypin, quercitrin, naringenin and quercetin from rhododendron cultivars [29] were extracted, separated and assayed. Out of latter proposals [11–29], determination of the naringin
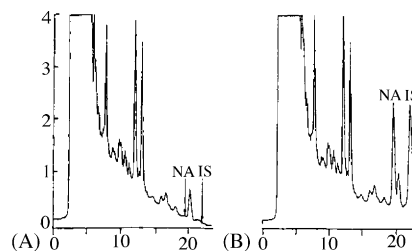

Fig. 4. HPLC chromatograms obtained at 324 nm: plasma extracts from blank urine (A) and from urine (B) spiked with naringin (NA: 142.5 ng mL^{-1}) and with hesperidin (IS: 166.3 ng mL^{-1}). Chromatographic conditions: column, Inertsil ODS-2, 5 μm, 250 mm × 4.6 mm i.d.; eluent, ACN–0.1 M ammonium acetate–triethyl amine (25:75:0.05, v/v), isocratic, pH 8, flow rate 1 mL min^{-1}. (With permission from [17].)

and naringenin of human plasma [16] and urine [17] and the analysis of grape anthocyanins [18] are worth to discuss in a more detailed manner.

Sample preparations from plasma and urine (Table 1 [16,17]) were carried out in a very conscientious manner. Extractions of test samples were accompanied with 'spiked' experiments ([17] Fig. 4), reproducibility percentages were calculated from six parallel extractions.

HPLC quantitation of red grape anthocyanins [18], was performed separately from the entire fresh grapes and from skins of the fresh grapes, subsequently to an optimized, exhaustive extraction study: seven conditions have been tested in triplicate, varying the composition of solvents and the time of extractions. Results of extraction studies were based on a convincing separation protocol, ensuring the complete resolution of seven anthocyanins (Fig. 5). Distribution of the flavonoid content of variously prepared extracts, in accordance with earlier experiences proved [18,19] that the use of neutral extraction solvent is to be preferred. The use of solvent containing up to 1% of 12 M HCl results in the partial hydrolysis of acetylated anthocyanins during extraction. As a consequence, authors [18,19] suggest the use of neutral sol-
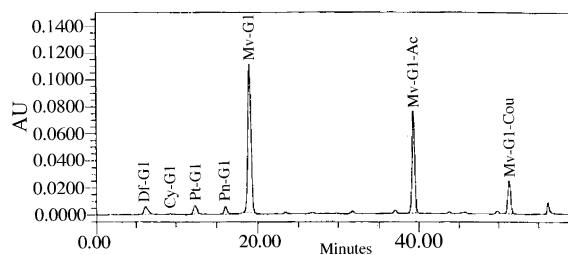
Fig. 5. HPLC chromatogram obtained at 520 nm from the skins of fresh grapes, extracted with 60% aqueous methanol, at 25 °C, for 4 h; peaks: delphinidin-3-*O*-glucoside (Df-Gl), cyanidin-3-*O*-glucoside (Cy-Gl), petunidin-3-*O*-glucoside (Pt-Gl), peonidin-3-*O*-glucoside (Pn-Gl), malvidin-3-*O*-glucoside (Mv-Gl), malvidin-3-*O*-acetylglucoside (Mv-Gl-Ac) and malvidin-3-*O*-*p*-coumarylglucoside (Mv-Gl-Cou); chromatographic conditions: column, Waters Nova-Pak C18 cartridge, 150 mm × 3.9 mm; eluent, water–ACN (60:40) linear gradient, adjusted with HClO$_4$ to pH 1.2, flow rate 1.5 mL min^{-1} at 32 °C. (With permission from [18].)

vents for extractions: both from grapes and from any other plant material containing acetylated anthocyanins.

4.2. HPLC–photodiode array [30–47] detection

In the community of 'poor' analytical chemists, ironically speaking, DAD is called as the substitute of MS. Unfortunately, this ironical aspect is not absolutely true but indicates the potential contribution of the DAD in the identification of unknown constituents: in a number of cases it serves as an excellent tool.

Based on the special spectral characteristics of flavonoids, their identification and quantitation applying the HPLC–DAD system provides higher certainty [30–48]; they were successfully and selectively investigated in several matrices such as:

(i) in biological fluids [32,48];
(ii) in juices/drinks of various canned fruits [31,37,47, 40,43,46], such as orange and grapefruit [31,37,47], seven red fruits [40], cranberry [43], raspberry, cranberry, apple and grape [46];
(iii) in extracts of fresh fruits [33,38,41] obtained from blueberries [33], from different apple varieties [38], from grape [41];
(iv) in extracts of vegetables [35,45] obtained from onion and celery [35] and from legumes [45];
(v) in tea infusions [39,42] as well as
(vi) in extracts of various herbs [30,34,36,44] obtained from the medicinal plants of Crataegus [30], Sideritis [34], Betula pendula and pubescens [36] and that of Semen Cuscutae [44].

Characteristic flavonoids were identified and measured in the extracts: either without hydrolysis in their initial conditions, in the form of glycosides and aglycones [31,32,34,36–41,44–48], or in differently prepared hydrolyzates as the corresponding aglycones [30,33,35,42,43].

4.2.1. Biological matrices

Identification of the metabolites of Ginkgo biloba [32], found in extracts of rat urine after 24 h administration proved the cleavage of the flavonol's skeleton: leading to the formation of various phenylalkyl acids and dihydroxy-benzoic acids.

Recovery studies of wine phenolics from human plasma [48] was performed in order to evaluate their bioavailability, i.e. to serve as epidemiological evidence demonstrating that wine consumption has beneficial effects on health. As a result of a sample pretreatment optimization relating to the types of extraction and deproteinization methodologies (based on recoveries of rutin, quercetin, kaempferol and *trans*-resveratrol), it has been shown that inhibition of plasma enzymatic activity, SPE and deproteinization with acidified ethanol is needed [131].

4.2.2. Canned fruit juices

The prominent flavonoids of citrus juice concentrates have been identified and quantitated, reporting in all three cases attractive reproducibility data [31,37,47]. In frozen orange and grapefruit concentrates from unknown origin [31], on the basis of absorbance at 283 nm, within 35 min, in order of listing in 100 g orange/grapefruit juice concentrates, 120/160 mg hesperidin along with 24/30 g/100 g narirutin (orange juices) and 200/200 mg naringin along with 62/68 mg narirutin (grapefruit juices) were found. In citrus juices from Spain and Israel, besides the three main flavanon glycosides (hesperidin, naringin, narirutin), although in lower concentrations, additional three flavanon glycosides (neohesperidin, didymin, poncirin) and six polymethoxylated flavons (sinensetin, hexamethoxyflavone, nobiletin, scutellarein, heptamethoxyflavone, tangeretin) were identified [37]. Latter flavonoids were quantitated, from a single solution, at characteristic UV maximum values (Fig. 6), within 60 min, with spectacular reproducibilities (pure juices: R.S.D. = 0.6–3.6%; juice concentrates: R.S.D. = 0.3–6.0%). Flavonoid composition of citrus juices from the Slovak market [47], prior to HPLC were filtrated only, contained three flavanone glycosides (naringin, hesperidin, neohesperidin) and one flavonol (quercetin). In accord with the composition of citrus juice concentrates [31], the main constituent of orange and grapefruit juices proved to be hesperidin (45–93 g L^{-1}; R.S.D. = 1.3–6.6%) and naringin (116–211 g L^{-1}; R.S.D. = 0.5–4.3%). Fresh pressed lemon juice contained neohesperidin (14 g L^{-1}; R.S.D. = 7.8%), exclusively. In addition, in orange juices naringin (2.0–7.0 g L^{-1}; R.S.D. = 3.4–7.0%), neohesperidin (5.0–11.0 g L^{-1}; R.S.D. = 5.0–6.6%) and quercetin (5–23 g L^{-1}; R.S.D. = 3.3–5.3), in grapefruit juices hesperidin (15–16 g L^{-1}; R.S.D. = 1.6–4.3%) neohesperidin (11–20 g L^{-1}; R.S.D. = 1.3–4.4%) and quercetin (7–12 g L^{-1}; R.S.D. = 2.6–4.7) were measured.

Several flavonoids were identified and measured in canned juices of various fruits [40,43,46]. From red fruit juices [40] forty, mainly anthocyanins, within 40 min, from cranberry [43], in its hydrolyzates, four flavonoids together with nine phenolic acids within 47 min, from raspberry, cranberry, apple and grape juices 10 organic acids and 21 flavonoids [46] within 90 min, on 250 mm × 4.6 mm [40,43] and on 150 mm × 4.5 mm columns were separated and determined.

4.2.3. Extracts of fresh fruits, vegetables

From Highbush blueberries [33], subsequently to the separation of its extract into the water-soluble and into the water-insoluble fractions, from these two fractions in total, 15 flavonoid glycosides and several phenolic acids were separated (column: 250 mm × 4.0 mm, 5 μm; flow rate, 1 mL min^{-1}, 22 °C), within 80 min, detected at 280 nm.

The peel and pulp fractions of different apple varieties were analyzed separately [38]. The highest level of phenolic compounds (Fig. 7A) were determined in the peel (catechin:
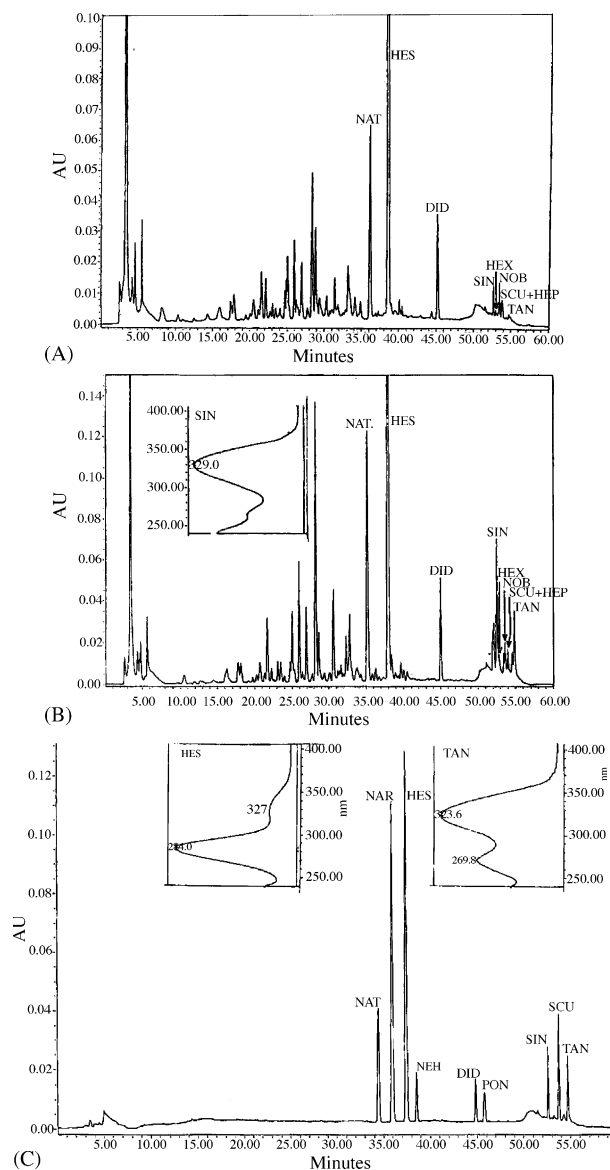
Fig. 6. HPLC chromatogram of flavanone glycosides and flavones. Chromatographic conditions: column, RP Altima, 5 μm, 250 mm × 4.6 mm i.d., UV detection between 260 and 350 nm; eluent, A: ACN, B: water–acetic acid (96:4, v/v); injected amount, 20 μL; flow rate, 1 mL min^{-1}, at 35 °C; (S) elution of standards; (A) Valencia, Spanish orange juice; (B) Ortanique (tangor) from Israel; peaks: Narirutin (NAT, 40) naringin (NAR, 50), hesperidin (HES, 5), neohesperidin (NEH, 2.5), didymin (DID, 2.5), ponciric PON, 2.5), sinensetin (SIN), scutallarein (SCU), tangeretin (TAN), hexamethoxyflavone (HEX), nobiletin (NOB), heptamethoxyflavone (HEP) (Fig. 4 S amounts of standards in parentheses, mg mL^{-1}). (With permission from [37].)

66–486 mg kg^{-1}, rutin: 136–671 mg kg^{-1}, procyanidin B2: 69–659 mg kg^{-1} fresh sample), while in the pulp, chlorogenic acid (28–357 mg kg^{-1} fresh sample) proved to be the main constituent (Fig. 7B).

Flavonoid content of grape berries [41] proved to be low: in its pulp only traces, in its peel rutin (187 mg/100 g), quercitrin (in traces), *trans*-resveratrol (15 mg/100 g) and quercetin (14 mg/100 g) were found.
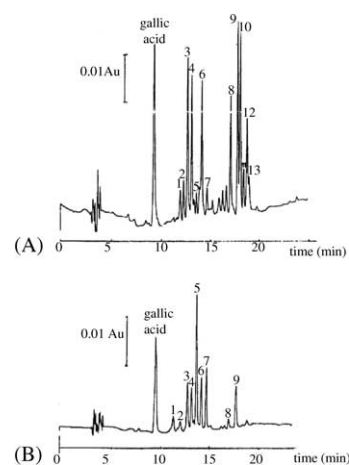

Fig. 7. HPLC chromatograms obtained from the peel (A) and from the pulp (B) of Golden apple. Chromatographic conditions: column, Nucleosil 120 C$_{18}$, 5 μm, 250 mm × 4.6 mm i.d., UV detection between 210 and 350 nm, monitoring at 280 nm; eluent, A: aqueous 0.01 M phosphoric acid, B: 100% methanol; injected amount, 20 μL; flow rate, 1 mL min^{-1}; peaks: 1 + 2, procyanidin B2; 3, (+)-catechin; 4, procyanin; 5, chlorogenic acid; 6, (−)-epicatechin; 7, caffeic acid; 8, phloretin derivative; 9, phloridzin; 10, rutin; 11, 12 and 13, flavonol glycosides (procianidin B1-B3 calculated as catechin; flavonol glycosides 11–13 calculated as rutin). (With permission from [38].)

In acid hydrolyzed extracts (2 M HCl, 4 h, 90 °C) of onion: one flavonoid (quercetin) in celery: three flavonoids (apigenin, luteolin and an unknown compound) were found (Fig. 8: upper traces unhydrolyzed, lower traces hydrolyzed extracts). Comparing the profile of chromatograms obtained from the unhydrolyzed and hydrolyzed samples, it is clear that the quantitation of aglycones in hydrolyzed sample is simpler.

Tea infusions [39,42] obtained from black, green and Jasmine leaves contain epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate in the concentration
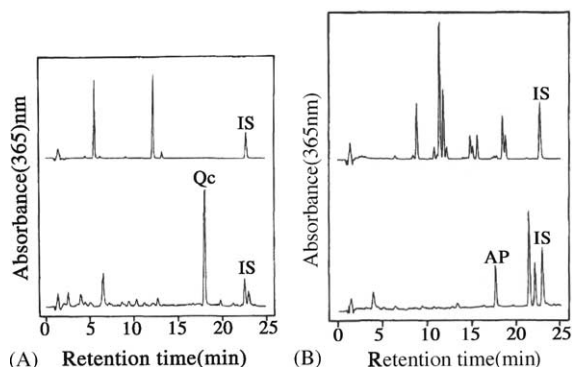
Fig. 8. HPLC chromatograms obtained from the extracts of white onion (A) and from celery (B), upper traces: unhydrolyzed, lower traces: hydrolyzed extract); chromatographic conditions: column, Symmetry C$_{18}$, 5 μm, 150 mm × 3.6 mm i.d., UV monitoring at 365 nm; elution: gradient of 15–35% ACN in water adjusted to pH 2.5 by TFA; flow rate 1 mL min^{-1}; peaks: (A) Qc, quercetin; IS, kaempferol as internal standard; (B) Ap, apigenin; Lt, luteolin; IS, isorhamnetin as internal standard. (With permission from [35].)

range of $10–130\,\text{mg}\,\text{L}^{-1}$ [39]. In the hydrolyzates of various green and black tea leaves infusions, [42] three flavonols were measured, in the concentration ranges, expressed in $\text{g}\,\text{kg}^{-1}$ dry leaves, as follows: $0.24–6.4\,\text{g}\,\text{kg}^{-1}$ (myricetin), $1.8–24\,\text{g}\,\text{kg}^{-1}$ (quercetin), and $1.6–9\,\text{g}\,\text{kg}^{-1}$ (kaempferol), respectively.

In the frame of an exhaustive study, the main phenolic classes of legumes were examined [45] as test samples lentils and beans were used. Benzoic and cinnamic acids, flavane-3-ols, flavones and flavonols were identified and quantitated with high recoveries (≥95%) and excellent reproducibilities (R.S.D. ≤ 7%).

Flavonoids in herb's extracts of medical importance [30,34,36,44] were investigated in their initial condition [30,34,36,44] and also subsequent to hydrolysis [30]. In Crataegus leave's hydrolyzate, mainly quercetin [30] was found. In extracts obtained from the entire plant of Sideritis (known as anti-inflammatory and anti-ulcer agent), 16 flavonoid species [34], from the leaves of Betula pendula and Betula pubescens, 45 flavonoid glycosides [36] and from the powdered seeds of Semen Cuscutae [44] (known as regulating the body's endocrine and immune system), five flavonoids were identified, with a recovery of 97–102%.

4.3. Separation of flavonoids applying postcolumn derivatization

The identification and quantitation of the derivatized flavonol content of beverages, based on the reaction between flavonols and *p*-dimethylaminocinnamal aldehyde was utilized in the postcolumn version [49], subsequent to their HPLC separation and UV detection at 280 nm, simultaneously. Thus, on the basis of this combined detection system, monitored before (A_{280}) and after derivatization (A_{640}), both the flavanol profile and the spectral characteristics upon the double monitoring served as an additional certainty of analyses.

4.4. Electrochemical detection [50–54]

Identification and characterization of flavonoids, because of the complexity of these compounds, often results in difficulties, relying exclusively on their UV or visible absorbencies. HPLC with amperometric detection can be a useful completion technique providing special selectivities. Diversities in electro active substituents on analogous structures may result in characteristic differences in their voltametric properties.

Baicalin, the 7-β-D-glucopyranoside of baicalein together with its aglycone, i.e. with baicalein (5,6,7-trihydroxyflavone), are excellent candidates for oxidation at a glassy carbon electrode to permit their selective electrochemical detection [50] providing high sensitivity (baicalin: $5\,\text{ng}\,\text{mL}^{-1}$ from $50\,\mu\text{L}$ plasma; baicalein: $2\,\text{ng}\,\text{mL}^{-1}$ from $100\,\mu\text{L}$ plasma).

In the analysis of phenolic and flavonoid compounds in various juice beverages [51] and in orange juice [52], also in comparison with HPLC/UV and HPLC/MS detections [52], the coulometric array detection proved to be a useful completion to the traditional techniques. Also, in the quercetin level determination in the cells of the immune system [53] and in validated assay of six metabolites originated from artichoke leaf extract in human plasma [54], HPLC combined with coulometric-array detection furnished spectacular selectivity and sensitivity.

4.5. Refractive index [55] and evaporative light scattering [56] detection

These two types of detections, as well known, are providing limited selectivity and sensitivity. However, both of them have been successfully used: the HPLC–refractive index (RI) system [55] in the quantification of leucocyanidin (3,3′,4,4′,5,7-hexahydroxyflavan) in unripe banana pulp, while the HPLC–evaporative light scattering detection one [56] in the determination of Ginkgo biloba flavonoids (Fig. 9).
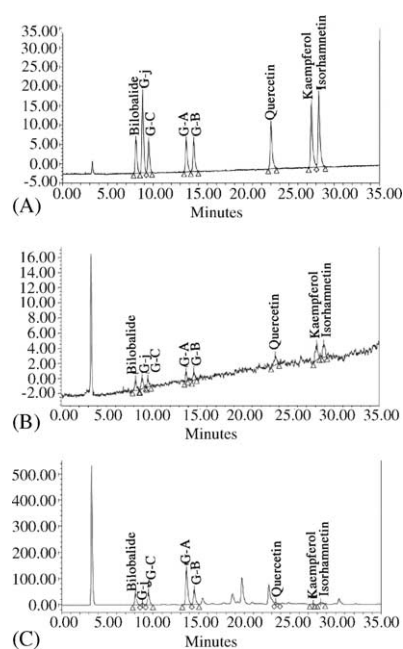
Fig. 9. HPLC–ELSD chromatograms obtained from model solutions (A and B) and from the acetyl acetate extract of Ginkgo biloba commercial product (C). Chromatographic conditions: column, Supelco Discovery RP-18, 5 μm, 250 mm × 4.6 mm i.d., ELSD: nebulization at room temperature, evaporation at 61 °C; elution: gradient; eluents, A: water containing 5% methanol and 0.05% TFA; B: methanol containing 0.05% TFA; flow rate, 1 mL min^{-1} at 20 °C; injected amount, 10 μL. Peaks: bilobalide, ginkgolide A, B, C, J: G-A, G-B, G-C, G-J; quercetin, kaempferol, isorhamnetin; amounts of stand in the injected 10 μL: 200 ng (200 ng); 20 ng (B) from all except B-J: 350 ng (A), 35 ng (B). (With permission from [56].)

Table 3
Identification and quantitation conditions of flavonoids from various matrices by HPLC/MS, HPLC/NMR and HPLC/CLND

The matrix	Separation conditions; column (Co), eluent (El), flow rate (Fl); injected (I)	Type of detection	Detection limit	Recovery, R.S.D. (%)	Number of compounds/time	Reference, year
Model studies						
–	Co: C$_{18}$ Guard-PAK, Waters; El: isocratic, ACN/H$_2$O) = 80/20 cont. 0.5% Ac.a.; Fl: 0.3 mL/min	DAD–IT (MS–MS, ESI/APCI); Q-TOF (MS–MS), PI/NI mode, both;	–	–	–/–: Vitexin, isovitexin, orientin, isoorentin	[67] 2001
Onion, elder-flower, wine	Co: poly(7-oxonorbornene-5,6-dicarboxylic acid-block-norbornene) coated silica, 150 mm × 4 mm, 7 μm, 50 Å; El: 25% ACN in 1% Ac.a. (v/v); Fl: 1 mL/min, 22 °C; I: 5 μL	DAD–(MS–MS) (ESI-PI)	UV: ng, MS: μg	–	5/5.5: Quercitrin, myricetin, quercetin, kaempferol, acacetin	[69] 2001
Wood pulp, wast water	Co: Phenomenex Luna C18, 150 mm × 4.6 mm; El (for the NI mode): A, 0.5% Ac.a.; B, Met; (for the PI mode): 65% Met/35% A (20% formic acid); Fl: 0.3 mL/min; I	(MS–MS) (ESI-PI/NI)	–	–	No data/14: monohydroxy flavons (5), daidzein, chrysin, apigenib, glangin, genistein, naringenin, kaempferol, catechin, quercetin	[70] 2001
–	Co: C18 Licchrospher, 250 mm × 4.5 mm, 5 μm; El: A, linear gradient of 0–30 min., 30–45% of Met; B, 45–97% B in A; Fl: 0.7 mL/min; I: 20 μL	DAD–(MS–MS) (ESI-NI)	12 ng	–	15/70 (aglycons only): luteolin, apigenin, genkwanin, chrysin, 7-OH flavon, quercetin, fisetin, kaempferol, galengin, kaempferid, eriodcty-ol, naringenin, isosakurametin, flavanon	[71] 2001
Vegetable tannins, water	Co: Hypersil ODS, 125 mm × 3 mm, 5 μm; El: A, H2O; B, Met (both cont,0.1% formic acid; Fl: 0.5 mL/min; 40 °C; I: 20 μL	DAD–(MS–MS) (ESI-NI)	2 ng	–	–/–: proanthocyanidin dimers-tetramers, catechin, gallocatechin (from tannin); ellagic-, gallic acid, gallotanins, ellagitannins (from chestnut)	[75] 2002
–	Co: Zorbax SB-18, 250 mm × 4.6 mm, 5 μm; El: 4 versions, 10 mM ammonium formate, 10 mM ammonium acetate (pH 4) Fl: 1.0 mL/, Met, ACN; I: 10 μL	(MS–MS)–(APCI-PI/NI) & (ESI-PI/NI) ion trap (IT); quadrupole (Qu)	1–50 ng	–	7-6/29: daidzein, daidzin, genistein, genistin, formononetin, ononin, biochanin A, sissotrin, hesperidin, heperitin, naringin, naringenin-7-glycoside, naringenin, rutin trihydrate, kaempferol	[79] 2003
Human plasma, urine	Co: Symmetry C18, 150 mm × 2.1 mm, 3.5 μm, or Spherisorb ODS2, Inertsil 250 mm × 4.6 mm, 5 μm; El: A, H$_2$O/ACN/Ac.a. = 96/2/2 v/v; B, ACN/Ac.a = 98/2 v/v; Fl: 0.3 mL/min, 30 °C; I: 20 μL	QqQ (MS–MS (ESI/PI/NI)	120 pg	–	7/60: epigallocatechin, epicatechin, epigallocatechin gallate, theafla-vin, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-digallate	[73] 2001

Fresh fruits, vegetables						
Sour orange	Co: Symmetry C18, 150 mm × 2.1 mm, 5 μm; El: A, H₂O cont 0.6% Ac.a.; B, Met; Fl: 0.2 mL/min; 45 °C; I: 3 μL	DAD–MS (ESI-PI)	–	–	11/35: synephrine, isonaringin, naringin, hesperidin, neohesperidin, naringenin, hesperitin, nobiletin, tangeritin, two not identidied	[60] 1997
Tea, apple/orange peel tomato, onion	Co: for hydrolyzates: Phenomenex RP C18, for glycosides: Purospher RP C18, both 250 mm × 4.6 mm, 5 μm; El: for hydrolysates: A, Met/H₂O = 30/70, v/v, cont 1% F. a.; B, Met; for glycosides: A, H₂O cont 1% F.a.; B, ACN; Fl: 1 mL/min; I: 20 μL	DAD–MS (API-NI)	50 μg	68–103 (recovery)	12/35 (aglycones; 2/60 glycosides	[62] 1998
Cultivated and wild berries	Co: LiChroCART, 125 mm × 3 mm, Purospher RP-18e, 5 μm; El: A, 1% F.a.; B, ACN; I: 20/5 μL (MS/DAD), Fl: 0.5 mL/min	DAD–MS–MS (IT-PI)	–	–	One by one/35 min: various glycosides of quercetin, myricetin, kaempferol	[64] 1998
Thirteen beers	Co: Phenomenex RP C18, 250 mm × 4 mm, 5 μm; El: A, 1% F.a.; B, ACN; Fl: 0.8 mL/min; I: 20 μL	DAD–MS–MS (APCI-PI): triple quadrupole = QqQ	20–200 pg	3.9–11.4 (R.S.D., %)	8/20: isoxanthohumol, xanthohumol, 2′, 4-dihydroxychalcone, 8-pre-nylnaringenin, 6-prenylnaringenin, 8- and 6-geranylnaringenins	[65] 1999
Apple, pear	Co: Phenomenex Aqua RP C18, 250 mm × 4 mm, 5 μm; El: A, 2% Ac.a. in water; B, 0.5% Ac.a in water/ACN = 50/50, v/vFl: 1 mL/min; 25 °C; I: 10 μL	DAD–MS (ESI-NI);	–	–	26/75: for compounds see Figure	[68] 2001
Artichoke waste	Co: Phenomenex Luna RP C18, 50 mm × 2.1 mm, 3.5 μm; El: A, 0.1% F.a. in water; B, 0.1% F.a in ACN; Fl: 0.4 mL/min; 25 °C; I: 10 μL	DAD–MS--S (turbo-IS-NI): QqQ	–	–	45 compouds identified, one by one: aglycones, glyosides and phenolic acids	[76] 2003
Soybean pods	Co: Vydac Multiring RP C₁₈, 250 mm × 4 mm, 5 μm; El: A, Ac.a. in water (pH 3); B, ACN; Fl: 1 mL/min	DAD–MS (APCI-PI);	–	–	7/105: glycosides of 7,4′-dihydroxyflavone, luteolin and apigenin,7,4′-dihydroxyflavone, apigenin-glycoside 2, luteolin, apigenin	[77] 2003
Fruit of cycl-anthera pedata	Co: Symmetry C18, 150 mm × 2.1 mm, 5 μm; El: A, 0.05% TFA in water; B, 0.05% TFA in Met; Fl: 0.3 mL/min; 45 °C; I: 20 μL	DAD–MS–MS (ESI-PI);	100–800 ng	–	11/60: 11 flavonid-glycosides	[78] 2003

I. Molnár-Perl, Zs. Füzfai / J. Chromatogr. A 1073 (2005) 201–227

213

Table 3 (*Continued*)

The matrix	Separation conditions; column (Co), eluent (El), flow rate (Fl); injected (I)	Type of detection	Detection limit	Recovery, R.S.D. (%)	Number of compounds/time	Reference, year
Herbs						
Saffron (*Crocus sativus*)	Co: Hewlett-Packard Hypersil ODS, 100 mm × 2.1 mm, 5 µm; El: linear gradient 10-100% Met in H2O cont.1% Ac.a.; Fl: 0.5 mL/min; I: 5 µL	DAD–MS (TSP/ESI-PI)	–	–	14/60: pirocrocin, pirocrocin-acidform, kaempferol diglycoside, trans-crocins2-5, trans-crocins2′, cis-crocins1-5, safranal	[57] 1995
Willow herbs (Epilobium)	Co: Waters Nova-Pak RP-18150 mm × 3.9 mm, 4 µm; El: linear gradient 10-25% ACN in H2O cont. 0.05% TFA.; Fl: 1 mL/min; I: 20 µL	DAD–MS (TSP-PI)	–	–	12/30: glycosides of quercitrin, guajaverin, hiperosid, isoquercitrin myricitrin, isomyricitrin	[58] 1995
Red clover	Co: Hewlett-Packard Hypersil ODS, 200 mm × 2.1 mm, 5 µm; El: A, H2O cont.0.25% Ac.a.; B, Met; Fl: 0.2 mL/min; 45 °C; I: 10 µL	DAD–MS (ESI-PI)	–	–	7/30: daidzin, genistin, isoquercitrin, daidzein, quercetin, genistein, biochanin A	[59] 1996
Cistus ladanifer L.	Co: LiCrosorb RP C_{18}, 250 mm × 24.5 mm, 5 µm; El: isocratic H2O/THF/Met/ACN = 56/22/16/6 cont.0.1 M ammonium acetate; Fl: 0.8 mL/min; I: 10 µL	DAD–MS (PB-PI)	–	–	8/50: apigenin, 3-methyl-kaempferol, 4′-(O) and 7-(O)methyl-apigenin, 3,4′- and 3,7-dimethyl-kaempferol, 7,4′-di(O)methyl-apigenin, 3,7,4′-*	[61] 1998
Hypericum perforatum	Co: Vydac Multiring RP C_{18}, 250 mm × 4 mm, 5 µm; El: A, Ac.a. in water (pH 3); B, ACN; Fl: 1 mL/min; I: 10 µL	DAD–MS (TSP/ESI-PI)	8–12 µg	<4.0 (R.S.D., %)	16/65: chlorogenic a. isomer, 3-O-p-coumaroylquinic a, chlorogenic a, rutin, hyper-osid, isoquercitrin, 3,3′,4′,5,7-pentahydroxyflavanon-7-glycoside, quercitrin, querce-tin, I3, II8 biapigenin, hypericin, hyperforins (3), pseudohypericin, adhyperforin	[63] 1998
Astragalus mongholicus	Co: Waters Symmetry C18, 150 mm × 2.1 mm, 5 µm; El: A, 0.05% TFA in water; B, 0.05% TFA in Met; Fl: 0.3mL/min; 45 °C; I: 10 µL	DAD–MS (ESI-PI)	0.2–1.2 ng	–	15/40: glycosides and glycoside malonates of calycosin, ononin, formonetin	[67] 2001
Helichrysum stoechas	Co: Waters Symmetry C18, 250 mm × 4.6 mm, 5 µm; El: A, 30 mM ammonium formate (pH 4.5)/ACN = 95/5, v/v; B, 30 mM ammonium formate (pH 4.5)/ACN = 5/95, v/v; Fl: 1 mL/min; 45 °C; I: 5 µL	DAD–MS (APCI-PI/NI);	500 ng	<2.5 (R.S.D., %)	13/26: phenolic acids and flavonoid-glycosides, see Figure 11	[72] 2001
Erigeron breviscapus	Co: Phenomenex Luna C18, 250 mm × 4.6 mm, 5 µm; El: ACN-H2O, from 10% to 40% ACN, v/v, within 30 min; Fl: 1 mL/min; 45 °C; I: 20 µL	DAD–MS (API-PI/NI); QqQ	0.55pg	>86% re-covery; <14% R.S.D.	7/46: apigenin-7-O-glucuronide, scutellarin, quercetin-3-O-glucuronide, apigenin, scutellarein, quercetin, baicalin	[74] 2001

h: human; pw: prewashed/conditioned; Fil: filtered through; H2O: distilled water; Met: methanol; Et: ethanol; El: elution with; vort mix: vortex mixing; centr: centrifugation; DMF: dimethylformamid; BHT: butylated hydroxytoluene, antioxidant; –: no data available; TBHQ: *tert*-butylhydroxyquinone (antioxidant); SDDC: sodium diethyl dithiocarbamate; AscA: ascorbic acid, stb: steam bath; PI: positive ion; NI: negative ion; Ac.a.: acetic acid; F.a.: formic acid; PB: particle beam; 3,7,4′-*: 3,7,4′-*-trimethyl-kaempferol; QqQ: triple quadrupole.
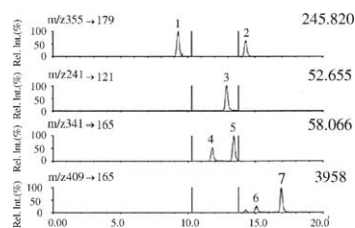
Fig. 10. HPLC–MS–MS a single chromatogram of multiple reaction ion monitoring, obtained from a direct analysis of commercial beer (vertical lines in the panels indicate the start of a new scanning period). (Chromatographic conditions in Table 2.) Peaks: 1, isoxanthohumol; 2, xanthohumol; 3, 2′,4-dihydroxychalcone; 4, 8-prenylnaringenin; 5, 6-prenylnaringenin; 6, 8-geranylnaringenin; 7, 6-geranyl-naringenin. (With permission from [65].)

4.6. Hyphenated detection techniques [57–81], such as on line HPLC–photodiode array detection–MS [57–79], HPLC–NMR [80], HPLC–chemiluminescence [81] and HPLC–photodiode array–MS–NMR [7] detection

HPLC coupled with various MS detectors proved to be the method of choice: in particular in the identification of flavonoids, unfortunately less in their quantitation. Out of 24 proposals cited, recovery and/or reproducibility data were found in six papers only (Table 3 [62,63,65,68,69,73,74]).

Excellent resolution, identification and quantitation was reported for the prenylflavonoids content of beers (responsible for the bitterness) by HPLC applying a triple quadrupole detector (QqQ) with an atmospheric pressure chemical ionization interface applying positive ionization (APCI-PI) [65]. (Further details: Table 3, Fig. 10.)

Model studies, providing comparison of different MS techniques and ionization modes, are of primary importance [67,69–71,75,79] furnishing useful informations to experts.

A basic study was carried out resulting in the differentiation of the C-glycosidic flavonoid isomers [67]. Possibilities of the quadrupole time of flight tandem mass spectrometry (Q-TOF-MS) and ion trap (IT) multiple stage mass spectrometry were evaluated under collision induced dissociation (CID). MS–MS spectra were evaluated performing various CID energy conditions. Data proved that low-energy LC–MS–MS(CID) spectra of C-glycosidic flavonoids differ from those obtained from high-energy measurements. However, the fragmentation profile of the MS–MS spectra of the C-6 and C-8 isomers are different, but differences do not give enough information. Specific loss of H_2O in the negative ionization mode (NI) from the C-6 glycosides can be used for discrimination. The most unambiguous differences appear in the spectra of the small precursor ions due to the different cleavages.

A new stationary phase and HPLC–DAD–MS detection were applied to the quantitation of phenolic acids and flavonoids, in total of 26 constituents of apple and pear samples [68]. Excellent resolution was obtained within 1 h elution time.

In order of a very fast (5 min) base-line separation of five common flavonoids, a special silica coating material was

used (Table 3), applying UV–MS–MS(ESI-PI) [69] detection. Comparative studies on the detection limits and reproducibility revealed in this special case that UV detection furnish about 10 times lower detection limit compared to the MS one, with the same reproducibility of the five flavonoids (Table 3: R.S.D., %).

The behavior of fourteen flavonoids, including monohydroxy to pentahydroxy substituted species, naringenin and galangin, applying MS–MS(ESI) detections, both in the NI/PI modes were studied [70]. Five different fragmentation processes were suggested depending on the energy conditions applied. Experiences have been utilized in measurement of genistein in low concentration in wood pulp, untreated wastewater and treated effluent from wood pulp mill [70].

The fragmentation patterns and the mechanism they are associated with are studied with six flavones (luteolin, apigenin, genkwanin, chrysin, 7-OH flavone), with five flavonols (quercetin, fisetin, kaempferol, galangin, kaempferid) and with four flavanones (eriodictyol, naringenin, isosakuranetin, flavanone) by MS–MS(ESI), in the NI mode [71]. Three different reaction mechanisms were proposed, proper for the discrimination of these three types of flavonoids. It has been demonstrated that following the retro Diels-Alder (RDA) fragmentation pathways several structurally informative anions appeared, highly specific of the NI mode. In addition, losses of unusual neutral molecules (CO, CO_2, C_3O_2, etc.) were also observed which seem to be characteristic to the NI mode and serve as a powerful complementary tool of the PI mode for the structural characterization of flavonoid aglycones.

An HPLC–DAD–MS method was developed for the identification and quantitation of the polyphenol oxidants of the Helichrysum stoecheas herb [72]. Ion trap detection (ITD) and APCI, both in the PI and NI modes, were tested: considerable differences between the two ionization modes were not found. For quantitation purposes, compounds from the DAD chromatograms were selected (further details in Table 3, Fig. 11).

Specific and sensitive analysis of theaflavins in biological fluids was measured by HPLC–DAD–MS–MS [73] (Further details in Table 3, Fig. 12).

An HPLC–MS–MS(QqQ) method was proposed for the research, identification and quantitation of apigenin, scuteallaerin, quercetin, baicalin and their corresponding glucuronides [74]. Quantitation was carried out on the basis of selective ion monitoring (SIM) with excellent sensitivity (further details in Table 3, Fig. 13).

A basic research study was carried out to identify the tannin composition of wattle and chestnut by MS–MS(ESI-NI) [75]. In the condensed wattle tannins, a series of proanthocyanidin dimers to tetramers, together with the monomer catechin and gallocatechin were identified. In the easily hydrolyzable tannin chestnut, a variety of gallotannins (mono-, di- and trigalloyl-glucose), elagitannins and the monomer ellagic and gallic acids were found. It has been repeat-
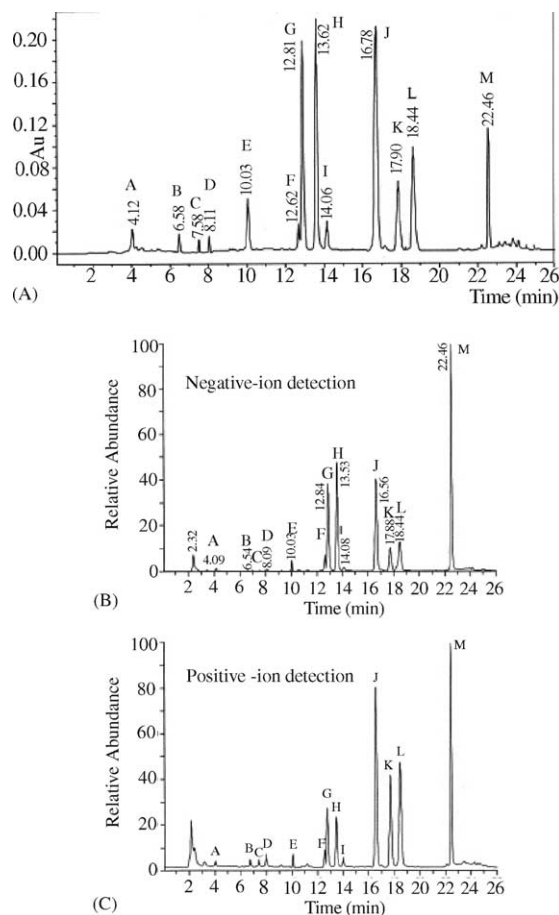
Fig. 11. HPLC–MS(APCI) TIC chromatograms obtained from Helichrysum stoechas' extract with negative- and positive-ion detections. (Chromatographic conditions in Table 3.) Peaks: A, neochlorogenic; B, cryptochlorogenic acids; C, unknown; D, unknown; E/F, dicaffeonylquinic acids; G/H, naringenin glucoside; I, quercetin-3-*O*-glucoside; J, kaempferol-3-*O*-glucoside; K/L, apigenin-glucoside; M, tetrahy-droxycalchone-2′-*O*-glucoside. (With permission from [72].)

edly stated that the use of NI mode is obligatory: PI mode leads to complex adduct formation that raises difficulties in identification.

Exhaustive optimization study was presented comparing the analytical advantages/disadvantages of the MS and tandem operations using ESI and APCI both in the PI and NI modes, applying a QqQ and an IT mass spectrometer [79]. As test samples 15 flavonoids were selected. Eluent composition and gradient program have been also optimized performing four different combinations: as organic modifier methanol and acetonitrile, as volatile buffer aqueous, 10 mM ammonium formate or 10 mM ammonium acetate were used, in all four combinations, at pH 4 (Table 3). Results obtained proved that in HPLC–UV all test compounds could be detected approximately with the same sensitivity. In the case of HPLC–MS responses, these varied up to two orders of magnitude, depending on the analytes and on the sets of conditions (Table 4, Fig. 14). In general, the methanol/ammonium formate eluent (pH 4) provided the highest MS responses in
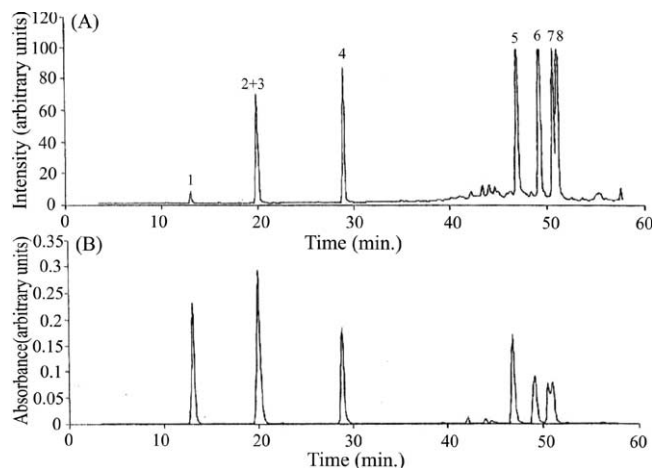

Fig. 12. HPLC–UV–MS chromatograms obtained from a mixture of black tea flavonoids (injected amounts, ∼70 pM μL⁻¹/compounds); chromatographic conditions in Table 3. (A) HPLC–MS–MS(ESI) of $m/z = 139$ parents; (B) UV trace at 280 nm. Peaks: 1, (−)-epigallocatechin; 2, (−)-epicatechin coeluting with 3, (−)-epigallocatechin gallate; 4, (−)-epicatechin gallate; 5, theaflavin; 6, theaflavin-3-gallate; 7, theaflavin-3′-gallate; 8, theaflavin-3,3′-digallate. (With permission from [73].)

the NI modes, in decreasing order of listing with APCI and ESI, respectively. Regarding the performance of the instruments, both QqQ and IT provided the same responses under each of the four ionization mode. The main ions proved
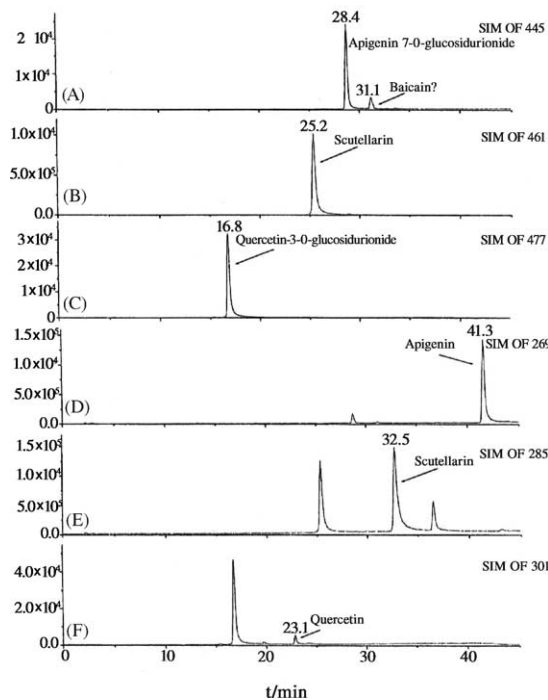

Fig. 13. HPLC–MS(API) chromatogram of the extract of *Erigeron breviscapus*, applying selected ion monitoring (SIM) in the NI mode. (Chromatographic conditions in Table 3.) Peaks: represent the molecular ions [M − H]⁻ of A, apigenin-7-*O*-glucuronide; B, scutellarin; C, quercetin-3-*O*-glucuronide; D, apigenin; E, scutellarein; F, quercetin. (With permission from [74].)

Table 4
Comparison of detection limit and reproducibility applying UV–MS–MS (ESI-PI) detection [69]

Flavon	Limit of detection[a]		R.S.D. (%)	
	UV[b] (ng)	MS (μg)	UV	MS
Quercitrin	0.3	0.18		
Myricetin	1.3	1.3		
Qquercetin	1.5	1.3	4.2	5.1
Kaempferol	0.6	1.5		
Acacetin	0.6	0.3		

[a] Signal-to-noise ratio of 3.
[b] Measured at 254 nm.

to be $[M - H]^-$ or, $[M + H]^+$, $[M + 45]^-$ and $[M - Gly]^-$ or $[M - Glu]^-$ (Gly, glycoside; Glu, glucoside).

An HPLC–UV–NMR approach was described for the fast identification of three flavonol glycosides in the leaves extract of Sorocea bomplandii, known of proven biological activity against gastritis and ulcers [80]. Quercetin-diglycoside, kaempferol di-, and triglycosides were identified applying the stopped flow technique.

The on line HPLC–chemiluminescence (CL) detection was developed for the screening of antioxidant flavonoids, such as (+)-catechin and (−)-epicatechin, present in green tea [81]. Determination of antioxidants is based on the decrease of CL intensity derived from luminol and superoxid anion radical $(O_2{}^-)$, i.e., from the enzyme reaction of xanthin oxidase with hypoxanthine. It means that a constant CL intensity as baseline is required and the antioxidants to be detected will be eluted as negative peaks corresponding to their antioxidant potential. The identity of the separated compounds were confirmed by MS(ESI) detection.
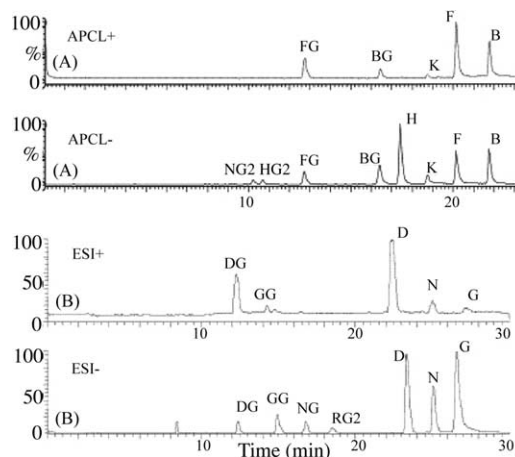

Fig. 14. HPLC–MS TIC chromatograms obtained on QqQ instrument, using APCI-PI and APCI-NI, separating eight compounds (A) and on an ion trap instrument, applying ESI-PI and ESI-NI, separating seven compounds (B) (chromatographic conditions in Table 3). Peaks: (A chromatograms) FG, ononin; BG, sissotrin; K, kaempferol; F, formononetin; B, biochanin A; NG2, naringin; HG2, hesperidin; (B chromatograms) Dg, daidzin; GG, genistin; D, daidzein; N, naringenin; G, genistein; Ng, naringenin-7-glycoside; RG2, rutin. (With permission from [79].)

Unambiguous structural information could be obtained by using the on line HPLC–DAD–MS–NMR technique [7]. The MS identification of flavonoid aglycones in mixture (Fig. 15A–D), such as diastereoisomers (A: catechin/epicatechin), or structural isomers with tiny differences (B: naringenin/apigenin, C: quercetin/fisetin, D: galangin/baicalein) by MS alone is not feasible, however, together with NMR informations due the special proton shifts are complete and satisfactory.

4.7. Identification/confirmation of flavonoids by MS and/or NMR techniques, subsequent to their HPLC–photodiode array detection analysis [82–90]

Twelve C-glycosylflavones of Passiflora incarnata (a widely used medical plant due to its sedative and tranquilizing properties) were identified and quantitated using an HPLC/DAD system [82]. One of these compounds (swertisin) was isolated at the first time and its structure elucidation applying off-line ^{1}H and ^{13}C NMR techniques.

Anthocyanins in hydrolyzates of cherries (Balaton and Montmorency) [83,87] and that of basil (*Ocimum basilicum* L.) [84] were isolated and their structure was elucidated off-line. Cherry anthocyanins were identified in recrystallised fractions by ^{1}H NMR spectroscopy [83] and by fast atom bombardment (FAB) mass spectrometry [87], while the structure of fourteen anthocyanins, isolated from basil [84], proved to be cyanidin based (eleven) and peonidin based (three) species.

Structure study of quercetin and its mono-, di- and triglucosides, in the hydrolyzate of red onion, were identified by homo- and heteronuclear NMR spectroscopy [85].

Seven flavonoids, isolated from the flowers of Nimphaèa caerulea were characterized by a combination of chromatography, homo- and heteronuclear two-dimensional NMR and MS(ESI) techniques [86].

Examination of the fractionated, non-volatile compounds of red wine was identified by FAB [88].

Dried flavonoids compiled from HPLC separated fractions, from the herb Amazonian cocae, were identified by ^{1}H NMR spectroscopy, their sugar constituents in hydrolyzates by GC–MS [89].

5. Separation of flavonoids by capillary electrophoresis [90–93] and by micellar electrokinetic capillary chromatography [94–98]

Separation of flavonoid-3-*O*-glycosides differing in their sugar moiety [90] and flavonoid-7-*O*-glycosides differing in their aglycones [94] were separated as borate complexes by CE [90] and by micellar electrokinetic capillary chromatography (MECC) [94], applying UV detection. In both cases [90,94], authors declared that CE and MECC gave higher efficiency, selectivity and speed compared to HPLC. To separate flavonoid-3-*O*-glycosides and flavonoid-7-*O*-glycosides
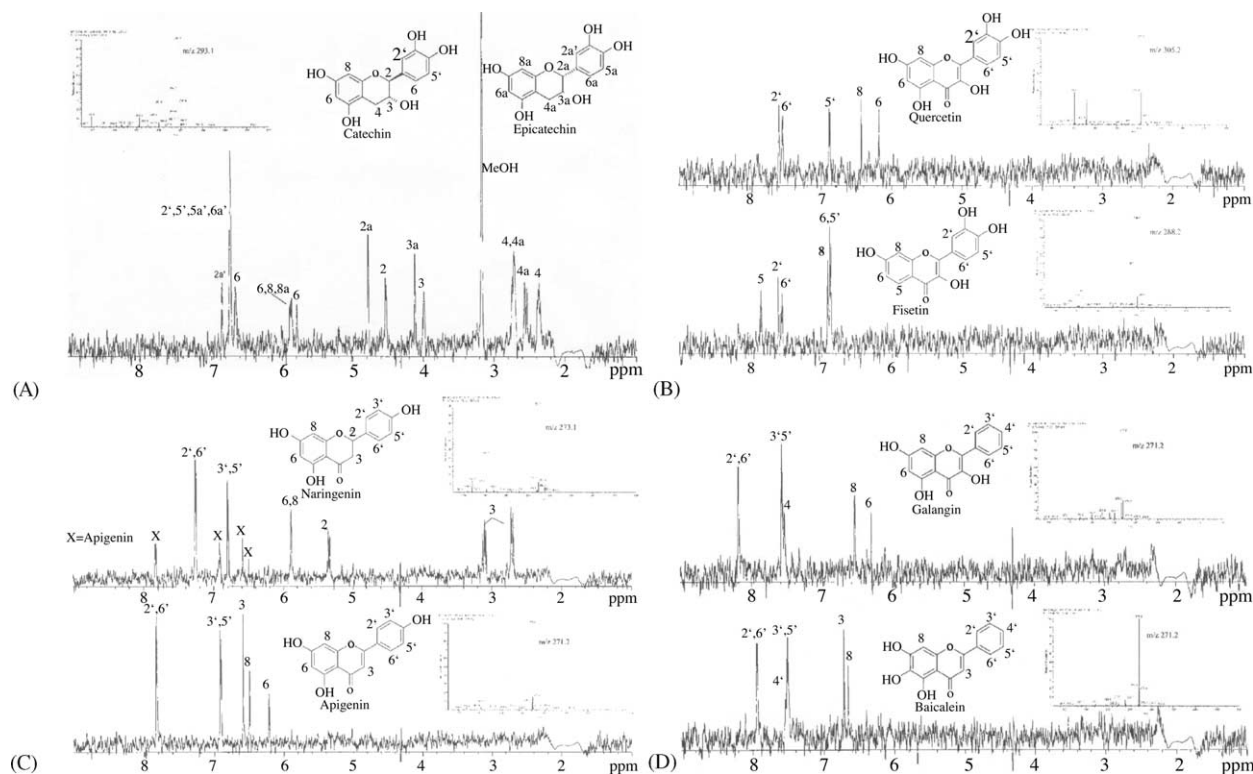
Fig. 15. HPLC–MS–NMR on-flow experiment carried out with the mixture of eight flavonoids (20 μg each, dissolved one by one, in methanol–ACN (1:1), in concentration of μg μL^{-1}); chromatographic conditions: column, Supelco Discovery RP-18, 5 μm, 250 mm × 4.6 mm i.d., MS(ESI-NI)–NMR detection; elution: gradient; eluents, A: 2H$_2$O; B: ACN; flow rate, 1 mL min^{-1}, splitted for 1/100: 10 μL min^{-1} (MS), 0.990 mL m^{-1} (NMR); spectra and chromatograms (A) MS and ^{1}H NMR spectra from the two-dimensional data set of the on flow experiment of catechin and epicatechin; (B) MS and ^{1}H NMR spectra from the two-dimensional data set of the on flow experiment of fisetin (top) and quercetin (bottom); (C) MS and ^{1}H NMR spectra from the two-dimensional data set of the on flow experiment of apigenin (top) and naringenin (bottom); (D) MS and ^{1}H NMR spectra from the two-dimensional data set of the on flow experiment of baicalein (top) and galangin (bottom). (With permission from [7].)

by CE 0.2 M borate buffer (pH 10.5), while for MECC separation of the flavonoid-7-*O*-glycosides, in the presence of sodium dodecyl sulfate, neutral conditions (pH 7.1) proved to be the optimum.

Tea catechin and theaflavins from tea infusions were analyzed, in parallel, by CE and HPLC [91] performing UV detections. Reproducibilities were approximately the same with the two methods (>90%). Analysis time for CE was three time faster (10 min versus 27 min), however, sensitivity was five times lower (500–5000 ng mL^{-1} versus 100–1000 ng mL^{-1}) in comparison to HPLC.

On the basis of a review [92] devoted to the evaluation of the analysis of food anthocyanins, comparing the performance of HPLC, HPLC–MS and CE, the following conclusion was drawn [92]: "The CE method permits the use of very small amounts of solvent, but does not offer the range of separation of complex samples that can be achieved with LC. Because the CE method uses much smaller samples, it does not offer any advantage over LC with respect of sensitivity. Each of these methods has a useful role in the analysis of anthocyanins in plant materials and products".

MECC separation and identification of flavonoids obtained from cruciferous plant [93], from honeys [95], from

Scute and Coptis herbs [96] and from green tea samples [97] were described.

In the frame of a basic research study, the MECC separation of 12 wine constituents was optimized [98]. The impact of buffer (pH), micelle-, electrolyte- and organic modifier concentrations (Fig. 16A and B) and the applied voltage were varied. Performing optimum conditions (SDS: 150 mM, electrolyte: 50 mM, methanol: 5%, pH 8.5, operating voltage: 20 kV) linear calibration curve was obtained for all analytes in the concentration range of 0.1–50 mg L^{-1}, reproducibility proved to be <6.8% R.S.D.

6. Separation of flavonoids by supercritical fluid, thin-layer and paper chromatography

Two isoflavones and one flavone from the fruits of Maclura pomifera were separated by supercritical fluid chromatography [99]. Leaf flavonoids of Lavandula and Sabaudia species were characterized by a combination of two-dimensional paper and liquid chromatography [100], while those of cruciferous species by a combination of liquid, paper and thin-layer chromatography [101].
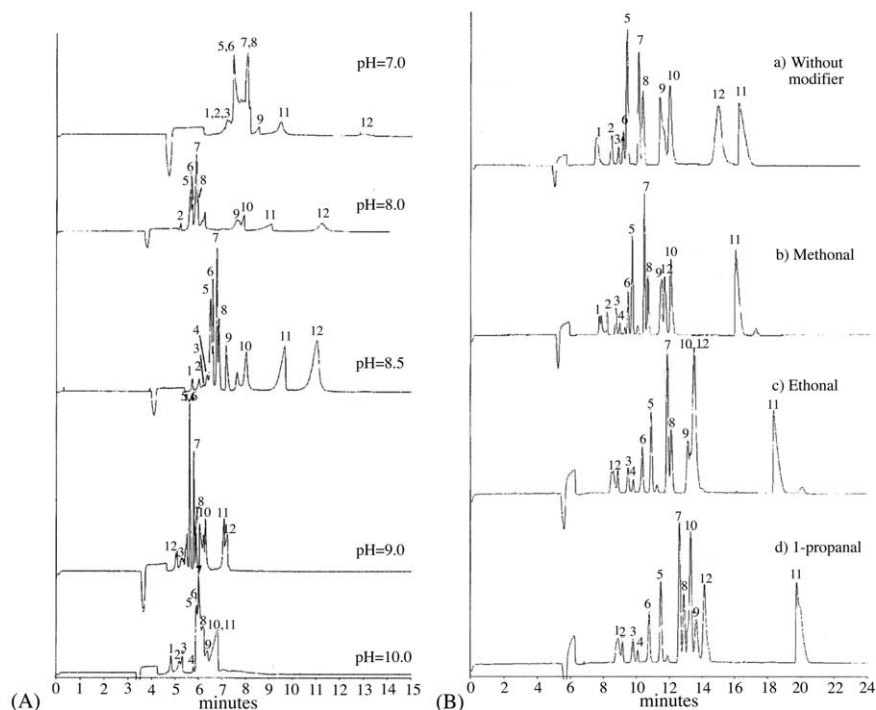
Fig. 16. Capillary electropherograms demonstrating the separation of 12 phenolic compounds as a function of the pH (A) and organic solvents (B). Conditions: 50 mM SDS as organic modifier; fused silica capillary [57(50) cm × 75 μm]; temperature, 25 °C; voltage, 20 kV; detection, 280 nm; hydrodynamic injection, 2 s. Peaks: 1, (+)-catechin; 2, (−)-epicatechin; 3, quercetin; 4, rutin; 5, protocatechuic aldehyde; 6, syringe aldehyde; 7, ferulic acid; 8, *p*-coumaric acid; 9, vanillic acid; 10, myricetin; 11, kaempferol; 12, caffeic acid. (With permission from [98].)

7. Identification and quantitation of flavonoids on the basis of their native fluorescence

A special attention is to be drawn on the native/self FL of flavonoids [102,103]. FL properties of flavonoids were examined applying TLC separation with fluorodensitometric detection [102]. Native fluorescence of fourteen flavone
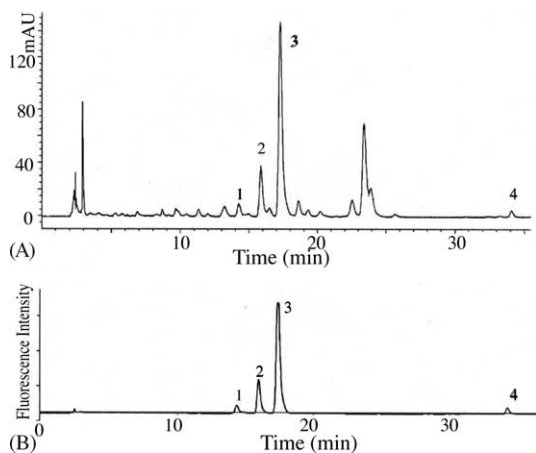

Fig. 17. HPLC chromatograms obtained from red clover extract by UV (A: 265 nm) and fluorescence (B: $E_x/E_m = 250/>450$ nm) detections. Conditions: column, Zorbax SB C$_{18}$ (250 mm × 4.6 mm, 5 μm), eluent: methanol/10 mM ammonium formate buffer, pH 4.0, flow rate: 1 mL min^{-1}; peaks 1, formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate isomer (FGM isomer); 2, ononin (FG); 3, FGM; 4, formononetin (F). (With permission from [103].)

and twenty-six flavonol type compounds were enhanced by their in situ reaction on the plate with diphenylboric acid 2-aminoethyl ester. It was recommended that in favor of reproducibility authentic reference standards, in appropriate concentrations, should be measured on the same plate. The correlation between fluorescence and molecular structure was given.

The native fluorescence of nineteen flavonoids was examined [103]. Out of test compounds only three isoflavonoids (daidzein, D; formononetin, F; ononin, FG) provided native fluorescence. Due to the large shifts between the excitation and emission wavelengths of the fluorescent isoflavones, from analytical point of view, outstanding selectivity can be obtained in comparison to UV detection (Fig. 17). In addition, also increased sensitivity was measured: limit of detections, at S/N = 3, in cases of FG and F, for the UV and FL detections, in order of listing proved to be 1 mg L^{-1} versus 0.1 mg L^{-1} and 0.5 mg L^{-1} versus 0.05 mg L^{-1}.

8. Separation of flavonoids by gas chromatography

Due to the relatively high-molecular mass and intrinsic feature of hydrophobic flavonoid aglycones and hydrophilic flavonoid glycosides, remaining on the safe and simpler side, the overwhelming part of chromatographic proposals is belonging to HPLC and/or to its related technologies. However, GC, primarily in the identification of aglycones as sily-

lated derivatives, completed by mass selective detection, can be regarded as pioneer techniques in the identification of flavonoids.

8.1. Separation and identification of flavonoids by GC without derivatization

These approaches [104–111], due to the high-molecular mass of flavonoids are surprising. It is based on a pyrolysis–field ionization mass spectrometry study [104]. Aiming to characterize plant materials resulted, in addition to pyrolysis fragments, also in the molecular ions of some flavonoid aglycones (kaempferol, epicatechin, pungenin, isorhamnetin) proving their volatility and their thermal stability under evaporation temperature (300–320 °C). This experience encouraged researchers to separate underivatized flavonoids (certainly aglycones, only), applying electron capture (ED) [105] and MS detections [106–111].

Evaluating data compiled in Table 5. It is clear that from quantitative analytical point of view these methods are of secondary importance: they are in shortage both of detection limit and those of reproducibility data, respectively.

The authors of the present paper are convinced that the GC analysis of underivatized flavonoid aglycones is to be accepted with limitations: appropriate to the performance of the method. They can serve as excellent identification possibilities (Fig. 18 [109]; Fig. 19 [110]). However, the statement
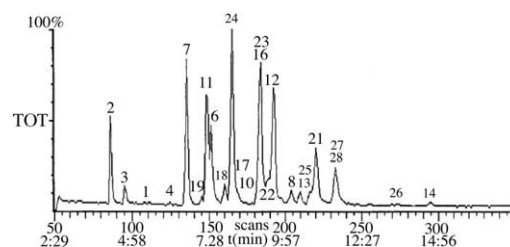

Fig. 19. GC–MS total ion chromatogram obtained from the combined flower head extracts of 10 different populations of Arnica angustifolia subspecies attenuata separated in their underivatized forms (chromatographic condition in Table 5). Peaks: 1, naringin; 2, naringin-7,4′-diMe-ether; 3, sakuranetin; 4, persicigenin; 5, eriodictyol-7,3′-diMe-ether; 6, acacetin; 7, apigenin-7,4′-diMe-ether; 8, pillion; 9, luteolin-7,3′,4′-triMe-ether; 11, pectolinaringenin; 12, salvigenin; 17, kaempferid; 18, rhamnocitrin; 19, kaempferol-7,4′-diMe-ether; 21, isorhamnetin; 22, rhamnazin; 23, 6-methoxykaempferol; 24, betuletol; 27, quercetagetin-6,3′,4′-triMe-ether; 28, eupalitin; peaks 10, 13–16, 20, 25 and 26 are not indicated. (With permission from [110].)

found in paper [111], i.e., "The results were compared to the respective *tert*-butyldimethylsilyl derivatized wood extractive profile, and it was stated that derivatization was unnecessary for the GC/MS analysis of the target compounds." cannot be accepted. Not at all on the basis of these two chromatograms (Fig. 20A and B) that should have serve as a confirmation of the above statement. Because, in contrary, they prove just the very opposite of it: neither the number of compounds nor their concentrations seem to be comparable.
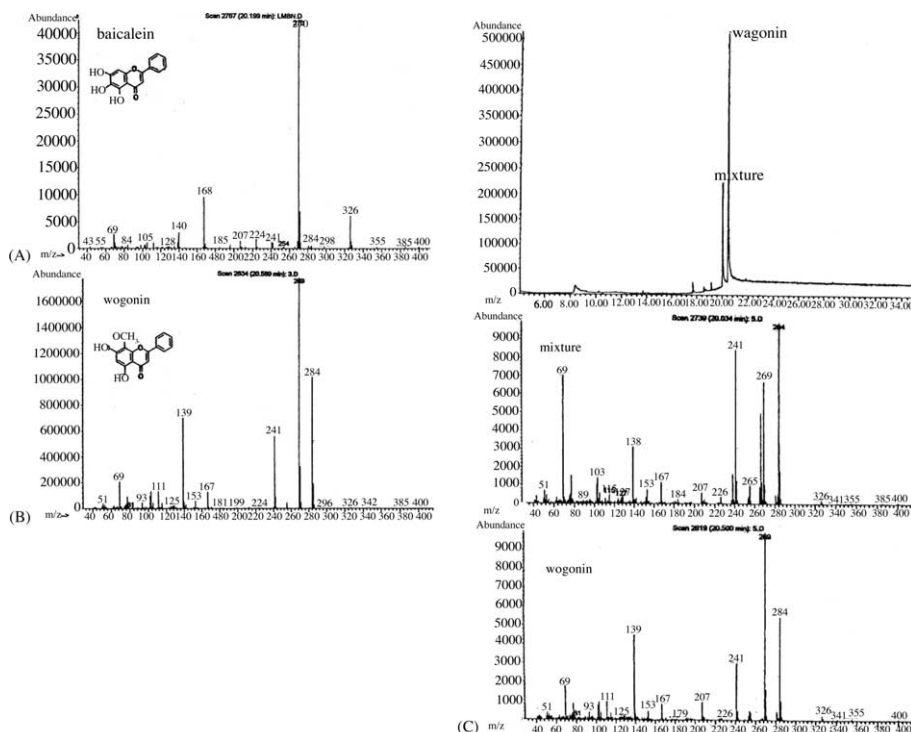
Fig. 18. Mass spectra of authentic baicalein (A) and wogonin (B), as well as the TIC profile of the components of a Scutellariae radix extract (C) obtained without derivatization by GC–MS (chromatographic conditions in Table 5). GC–MS spectra of baicalein (A: retention time, 20.20 min, $[M]^{\pm} = m/z$ 270) and wogonin (B: retention time: 20.59 min; $[M]^{\pm} = m/z$ 284; $[M - CH_3]^+ = 269$); C: TIC profile of the radix extract (upper trace), as well as spectra of the mixture of baicalin, baicalein and oroxyclin-A (middle trace) and wogonin (bottom trace). (With permission from [109].)

Table 5
Identification and quantitation of flavonoids by gas chromatography as silyl derivatives

The matrix	Extraction (E); hydrolysis (Hy); (Co:); injector/detector temperature, °C (I:D:); derivatization (Der:); gradient program (G:)	Detection			Number of compounds/time	Reference
		Detection	LOD	R.S.D. (%)		
Propolis	E: 1 g + 20 mL 70% Et (overnight amb temp) → evap; Hy: –; Der: 1.5 mg extr + 95 µL BSTFA (65 °C, 30 min); Co: 9 m × 025 mm [123], 20 m × 0.30 mm [130]; Inj: 1 µL; I: 300 °C; D: 320 °C; G: 80–280 °C (20 °C/m) 280–300 °C (2 °C/m) [123], 40–250 °C (40 °C/m) → 390 (12 °C/m), hold 20 m [128]	GC–FID [123], GC–MS [128]	640 ng	<9.2	4/30: pinocembrin, galangin, caffeic acid, β-phenyl-ethyl caffeate	[123,128]
Eurasian Sedum, Sempervirum	E: 10 g dried leaves hog by 40 g AC centr, supern evap. aqueous sol dil by H₂O to 10 g, washed; Hy: extr + 10 mL 2 M HCl (100 °C, 1 h) evap, extr by Et₂O (3 mL × 15 mL); Der: dried extract + 0.5–1 mL PYR → 100 µL + 100 µL BSA (70 °C, overnight); Co: 10 m × 0.32/0.25 (FID/MS; I: 250 °C; D: 320 °C (FID) G: 125–325 (4 °C/m)	GC–FID GC–MS	200 ng	<6	10/50: kaempferol, herbacetin, sezangulaterin, quercetin, gossypetin, coniculatusin, isorhamnetin, limocitrin, myricetin, hibiscetin	[124]
Gingko biloba's flavonoids in h. urine	E: susequent to Hy: 1 mL urine + 0.5 mL 3 M HCl (80 °C, 1 h), or β-glucuronidase + 1 mL buffer (pH 8) → E: SPE (Table 1); Der: residue + 0.2 mL BSA (70 °C, 30 m); Co: Restek RTX-5 (30 m × 0.32 mm); I: 250 °C; trl: 280 °C; G: 160–290 °C (20 °C/m), →320 °C (5 °C/m)	GC–MS	10 pg	<9.4	2/15: quercetin, kaempferol	[125]
Gingko biloba's flavonoids in pharmaceuticals	E: 40 mg extract + 5 mL 1 M HCl in 20% Met (85 °C, 1 h); mixture extr with 5 mL EtAc (1 m vortex, 5 m sonic, 10 m centr) → 50 µL organic layer + 250 µL DMF + 250 µL BSTFA cont 1% TMCS (115 °C, 45 m); Co: HP Ultra 1 (20 m × 0.20 mm); I: 275 °C; trl: 290 °C; G: 80–245 °C (25 °C/m) held for 25.5 m then 270 °C (60 °C/m) held for 8 m	GC–MS	0.5–2.5 ng	<7.8	7/50: bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, kaempferol, isorhamnetin, quercetin	[127]
Biological fluids	E: 100 µL serum/urine + 0.8 mL EtAc, then 0.5 mL EtAc → centr (5 m, 3500 × g); EtAc layer fil. (Na₂SO₄); Der: 100 µL EtAC + 100 µL BSTFA (vortexing 30 s, 70 °C, 1 h); Co: DB-5 I: 280 °C; D: 280 °C; G: 120(2 m) ramped to 300 °C (20 °C/min), at 300 °C (6m)	GC–MS	5 pg	6.9–18.5	4/17: *trans*-resveratrol, catechin, fisetin, quercetin	[126]

h: human; pw: prewashed/conditioned; Fil: filtered through; H₂O: distilled water; Met: methanol; Et: ethanol; El: elution with; vort mix: vortex mixing; centr: centrifugation; DMF: dimethylformamid; BHT: butylated hydroxytoluene, antioxidant; –: no data available; TBHQ: *tert*-butylhydroxyquinone (antioxidant); SDDC: sodium diethyl dithiocarbamate; AscA: ascorbic acid, stb: steam bath; PI: positive ion; NI: negative ion; Ac.a.: acetic acid, F.a.: formic acid; PB: particle beam; 3,7,4′-*: 3,7,4′-*-trimethyl-kaempferol; QqQ: triple quadrupole; Dil: diluted; EC: electron capture; Ac: aceton; trl: transfer line; IT: ion trap; M: manifold; amb: ambient temperature; TBDMS: *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamid; Et₂O: diethyl ether; BSA: *bis*-trimethylsilyl acetamide; PYR: pyridinr; EtAc: ethyl acetate.
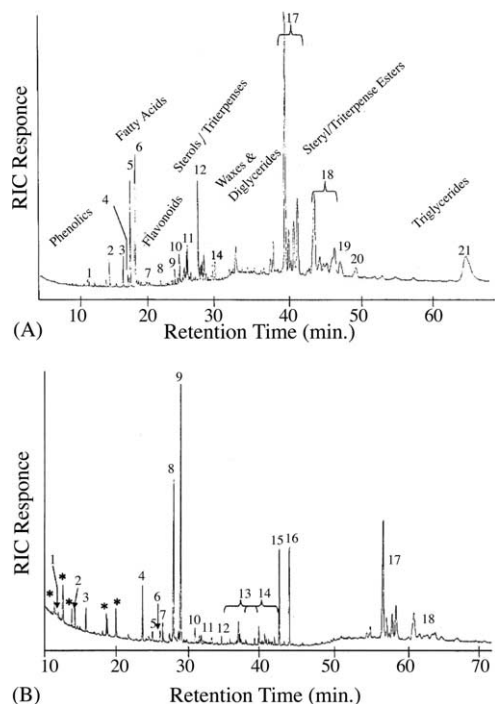
Fig. 20. GC/MS total ion chromatograms obtained from aspen wood extracts without derivatization (Fig. 18A, 1 µl at 5.00 mg mL^{-1}) and as TBDMS derivatives (Fig. 18B, 2 µl at ∼0.83 mg mL^{-1}) depending on derivatization yield. (Chromatographic conditions in Table 5.) Peaks (A) 1, 4-hydroxybenzoic acid; 2, 3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol; 3, hexadecanoic acid; 4, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenol; 5, internal standard (heptadecanoic acid); 6, (Z,Z)-9,12-octadecadienoic acid; 7, eicosanoic acid; 8, docosanoic acid; 9, 4′,5-dihydroxy-7-methoxyflavanone; 10, 4′,5,7-trihydroxyflavanone; 11, stigmast-5-en-3β-yl acetate; 12, stigmast-5-3β-ol; 13, 24-methylcycloart-24,(24′)-en-3β-ol; 14, unidentifed sterols/triterpenes; 15, various waxes; 16, unidentified steryl/triterpene esters; 17, hexadecanoate (C16:0) steryl/triterpene esters; 18, octa-decanoate (C18:0) steryl/triterpene esters; 19, unidentifed triglyceride; 20, tirucalla-7,24-diene-3β-yl-eicosanoate; 21, (9Z,12Z)-glycerol-tri-9,12-octadecadienoate. Peaks (B) *Silylation contaminants (seen in blank); TBDMS derivatives of 1, benzoic acid; 2, mono-saccharide; 3, unknown; 4, 4-hydroxybenzoic acid; 5, monosaccharide; 6, 1,9-nonanedionic acid; 7, hexadecanoic acid; 8, internal standard; 9, (Z,Z)-9,12-octadecadienoic acid; 10, unknown; 11, eicosanoic acid; 12, docosanoic acid; 13, C$_{23}$:0–C$_{26}$:0 fatty acids; 14, various underivatized and some derivatized srerols/triterpenes; 15, 4′,5,7-trihydrox-yflavanone; 16, 4′,5-dihydroxy-7-methoxy-flavanone; 17, underivatized hexadecanoate steryl/triterpenes esters; 18, underivatized octadecanoate steryl/triterpene esters. (With permission from [111].)

8.2. Separation, identification and quantitation of flavonoids as derivatives

8.2.1. GC of flavonoids as their methylated and ethylated derivatives

The early GC–MS study [112] of perdeuteromethylated flavonoid aglycones are of theoretical importance. Methylation was carried out with C_2H_3I and NaH in dimethylformamide. This method provided information about the sugar sequence, their interglycosidic linkages and the sugar attachment to the aglycone.

Flavonoid content of the New Zealand sourced propolis was identified and quantitated from a tincture solu-

tion, in parallel by HPLC and by GC/MS as methylated and ethylated derivatives [113]. HPLC was the preferred method because of the low response factors of flavonoids in GC/MS.

8.2.2. GC of flavonoids as their silyl derivatives

Pioneer gas chromatographic quantitation of flavonoids provided precious experiences [114–116]. The simultaneous extraction/derivatization process of rutin [114], main constituent in the pulverized sample of Sophora japonica, was carried out by heating the sample in pyridine at 120 °C, with bistrimethylsilyl acetamide (BSA) for 4 h. Extraction and derivatization of the same sample was incomplete at 70 °C, even after 7 h, followed to stand the reaction mixture for further two days at room temperature.

Model GC–MS fragmentation pattern of anthocyanidins [115], silylated by hexamethyldisilazane/trimethylchlorosilane (HMDS/TMCS) revealed the formation of quinolin type compounds: due to the nucleophylic attack of an NHSi(CH$_3$)$_3$ group, at position 2 and the rearrangement of the (CH$_3$)$_3$ Si group from the nitrogen atom to the pyranyl oxygen atom.

Anthocyanins of the fruit Vaccinum myrtillis that contain up to 15 anthocyanins (3-O-arabinosides, 3-O-glucosides, 3-O-galactosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin) have been identified and quantitated as their trimethylsilyl (TMS) derivatives by GC–FID and by GC–MS [116].

Exhaustive GC/MS identification and quantitation studies of various bud exudates of Populus (P) species [117–122] (P. lasiocarpa [117], P. angustifolia [118], P. trichocarpa [119], P. euphratica [120], P. ciliata [121]) leaded to their chemotaxonomy [122].

Flavonoids of propolis [123,128] were identified and quantitated subsequent to their derivatization with bistrimethylsilyl trifluoroacetamide (BSTFA) by FID and by MS detection (Table 6). Reproducibility data and detection limit values were given to FID, only [123]. GC–MS investigation of the two Bulgarian propolis was focused on the measurement of their antibacterial activity due to their flavonoid contents [128].

Flavonoids from the vegetative parts of twenty-nine Eurasian Sedum and 34 Sempervivum species have been identified from their hydrolyzates (Table 6) [124]. Quantitative analysis of flavonoids obtained by GC–MS after trimethylsilylation were the basis of multivariate data analysis. Principal component analysis of the whole data set distinguishes Sedum and Sempervivum as separate groups.

Flavonoid content originating from the leaf extract of Ginkgo biloba, in human urine [125] and in pharmaceutical preparations [127] has been identified in extracts immediately [125] and subsequent to hydrolysis [125,127]. Detection limit (Table 6) was considerably better in human urine (40 pg) compared to pharmaceutical preparation (0.5–2.5 ng). Hydrolysis of urine samples resulted in markedly higher quercetin and kaempferol content (Fig. 21) due to the fact that these two

Table 6
Identification and quantitation of flavonoids by gas chromatography as silyl derivatives

The matrix	Extraction (E); hydrolysis (Hy); (Co:); injector/detector temperature, °C (I:D:); derivatization (Der:); gradient program (G:)	Detection			Number of compounds/time	Reference, year
		Detection	LOD	R.S.D. %		
Propolis	E: 1 g + 20 mL 70% Et (overnight amb temp) → evap; Hy: –; Der:1.5 mg extr + 95 μL BSTFA (65 °C, 30 min); Co:9 m × 025 mm [123], 20 m × 0.30 mm [130]; Inj: 1 μL; I: 300 °C; D:320 °C; G: 80–280 °C (20 °C/m)280–300 °C (2 °C/m) [123], 40–250 °C (40 °C/m) → 390 (12 °C/m), hold 20 m [128]	GC–FID [123]	640 ng	<9.2	4/30: pinocembrin, galangin, caffeic acid, β-phenyl-ethyl caffeate	[123,128] 1992, 2003
		GC–MS [128]	–	–		
Eurasian Sedum, Sempervirum	E: 10 g dried leaves hog by 40 g AC centr, supern evap. Aqueous sol dil by H$_2$O to 10 g, washed; Hy: extr + 10 mL 2 M HCl (100 °C, 1 h) evap, extr by Et$_2$O (3 mL × 15 mL); Der: dried extract + 0.5–1 mL PYR → 100 μL + 100 μL BSA (70 °C, overnight); Co: 10 m × 0.32/0.25(FID/MS; I: 250 °C; D:320 °C (FID) G: 125–325 (4 °C/m);	GC–FID GC–MS	200 ng	<6	10/50: kaempferol, herbacetin, sezan-gulaterin, quercetin, gossypetin, coni-culatusin, isorhamnetin, limocitrin, myricetin, hibiscetin	[124] 1996
Gingko biloba's flavonoids in h.urine urine	E: susequent to Hy: 1 mL urine + 0.5 mL 3 M HCl (80 °C, 1 h), or β-glucuronidase + 1 mL buf-fer (pH 8) → E: SPE (Table 1); Der: residue + 0.2 mL BSA(70 °C, 30 m); Co: Restek RTX-5 (30 m × 0.32 mm); I:250 °C; trl: 280 °C; G: 160–290 °C (20 °C/m), →320 °C (5 °C/m)	GC–MS	10 pg	<9.4	2/15: quercetin, kaempferol	[125] 1999
Gingko biloba's flavonoids in pharmaceuticals	E: 40 mg extract + 5 mL 1 MHCl in 20% Met (85 °C, 1 h); mixture extr with 5mL EtAc (1 m vortex, 5 m sonic, 10 m centr) → 50 μL organic layer + 250 μL DMF + 250 μL BSTFA cont 1% TMCS (115 °C, 45 m); Co: HP Ultra 1 (20 m × 0.20 mm); I: 275 °C; trl: 290 °C; G: 80–245 °C (25 °C/m) held for 25.5 m then 270 °C (60 °C/m) held for 8 m;	GC–MS	0.5–2.5 ng	<7.8	7/50: bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, kaempferol, isorhamnetin, quercetin	[127] 2003
Biological fluids	E: 100 μL serum/urine + 0.8 mL EtAc, then 0.5 mL EtAc → centr (5 m, 3500 g); EtAc layer fil. (Na$_2$SO$_4$); Der:100 μL EtAC + 100 μL BSTFA (vortexing 30 s, 70 °C, 1 h); Co: DB-5 I: 280C; D:280 °C G: 120(2 m) ramped to 300 °C (20 °C/min), at 300 °C (6 m)	GC–MS	5 pg	6.9–18.5	4/17: *trans*-resveratrol, catechin, fisetin, quercetin	[126] 2001

h: human; pw: prewashed/conditioned; Fil: filtered through; H$_2$O: distilled water; Met: methanol; Et: ethanol; El: elution with; vort mix: vortex mixing; centr: centrifugation; DMF: dimethylformamid; BHT: butylated hydroxytoluene, antioxidant; –: no data available; TBHQ: *tert*-butylhydroxyquinone (antioxidant); SDDC: sodium diethyl dithiocarbamate; AscA: ascorbic acid, stb: steam bath; PI: positive ion; NI: negative ion; Ac.a.: acetic acid; F.a.: formic acid; PB: particle beam; 3,7,4′-*: 3,7,4′-*-trimethyl-kaempferol; QqQ: triple quadrupole; Dil: diluted; EC: electron capture; Ac: aceton; trl: transfer line; IT: ion trap; M: manifold; amb: ambient temperature; TBDMS: *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamid; Et$_2$O: diethyl ether; BSA: *bis*-trimethylsilyl acetamide; PYR: pyridinr; EtAc: ethyl acetate.

I. Molnár-Perl, Zs. Füzfai / J. Chromatogr. A 1073 (2005) 201–227
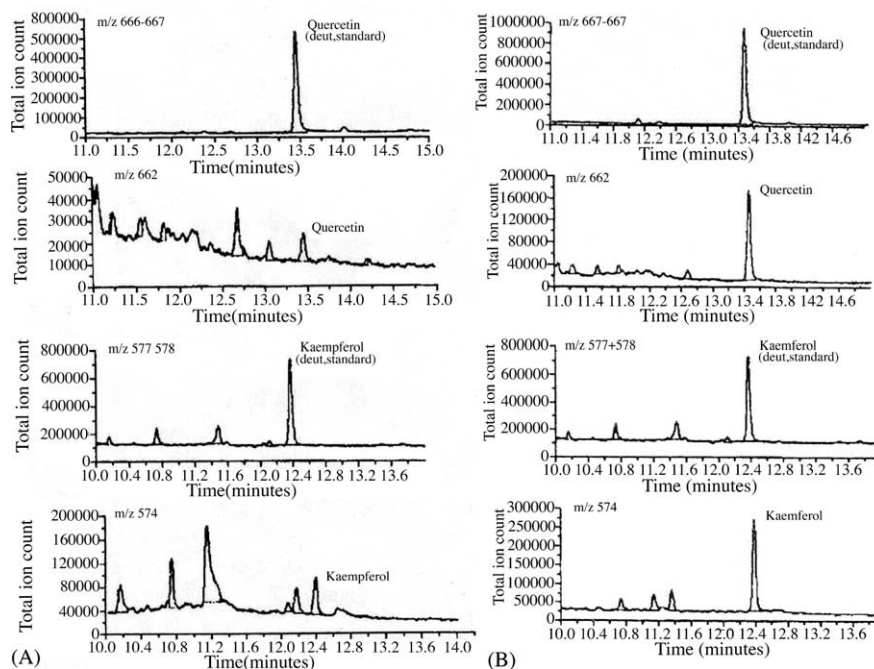
223

Fig. 21. Selected ion profile chromatograms of untreated (A) and hydrolyzed (B) urine samples monitored by the molecular ions of TMS-quercetin ($[M]^{\pm} = m/z = 662$) and that of the TMS-kaempferol ($[M]^{\pm} = m/z = 574$) (chromatographic conditions in Table 6). (With permission from [125].)
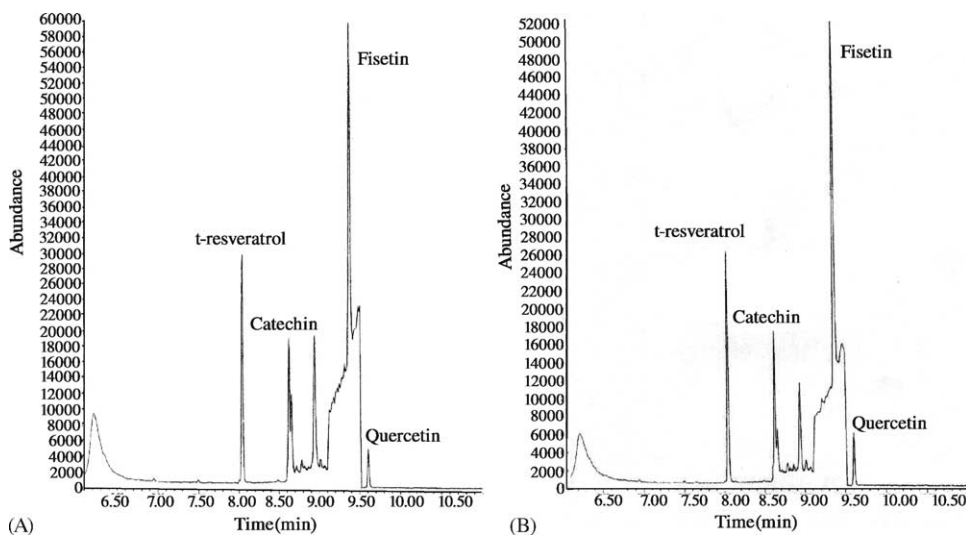

Fig. 22. Selected ion profile chromatograms of the TMS–derivatives of *trans*-resveratrol $m/z = 444 + (445, 446)$, (+)-catechin ($m/z = 369 + (368, 370)$, fisetin ($m/z = 471 + 399, 560$), quercetin ($m/z = 647$ (648, 649) obtained from human serum (A) and from human urine (B), respectively (chromatographic conditions in Table 4). (With permission from [126].)

aglycones are present in the urine as glucuronides (Fig. 21A and B) [125].

An ultra-sensitive GC–MS method was developed for the quantitation of catechin, quercetin and resveratrol in biological fluids [126] applying fisetin as internal standard (Fig. 22A and B). Selective ion monitored elutions based on the quantitation of target ions as follows: $[M + 3TMS]^+ = m/z$ 444 (*trans*-resveratrol), $[M + 5TMS\text{-}248\text{-}CH_3]^+ = m/z$ 369 (catechin) and $[M + 5TMS\text{-}CH_3]^+ = 647$ (quercetin), respectively (m/z 248, *O*-dihydroxybenzene2TMS).

9. Conclusion

Based on the overview of more than 500 papers and on the detailed critical evaluation on 133, it became confirmed that preliminary preparation steps, such as isolation/extraction, are of primary importance influencing the reliability and reproducibility of the analysis.

(1) In order to isolate/extract flavonoids from different natural matrices several classical and recent techniques have

been tested. As final conclusion, it can be stated that the traditional approach to find optimum condition, by varying parameters one by one, remains an illusion, only: it means, the process itself, the composition of extracting agent to the analyte, the time, the temperature, etc., that could have been accepted in general, were not found. Optimum extraction/isolation conditions are dependent on the compound(s) and on the matrix to be isolated from.

(2) On the hydrolysis step of flavonoids, being present in natural matrices partly as free aglycones, partly in the form of glycosides, special emphasis is to be put, and, two ways can be followed:

  (i) Chromatographic separation of the flavonoid content of extract, without hydrolysis, expected to be resulted in the identification and quantification of the free and sugar moiet(y/ies) containing flavonoids, simultaneously, in the presence of each other.

  (ii) Separation of flavonoids from a hydrolyzed extract provides decreased number of compounds to be determined, resulting in better resolution and improved characterization of the flavonoid aglycones. Certainly, in this case free aglycones and aglycones initially in glycosidic linkages cannot be distinguished.

(3) For the separation, identification and quantitation of flavonoids chromatographic techniques are the method of choice: in order to get optimum information detection should be as hyphenated as possible.

(4) Simple combinations, such as HPLC–UV, HPLC–DAD, HPLC–ELSD, CE–DAD, GC–FID, might be also useful and informative.

(5) However, the use of HPLC–MS and GC–MS proved to provide convincing and satisfactory results, in all cases performed.

  (i) HPLC–MS was applied, in the overwhelming part of proposals, the HPLC–DAD–MS combination. For quantitation purposes, the DAD chromatograms were preferred due to the fact that the MS responses of flavonoids vary by two orders of magnitude, while their DAD ones only by a factor of three. Consequently, HPLC–MS was utilized primarily for identification purposes, only.

  (ii) Regarding the performance of the MS detector systems, quadrupole and ion trap, as well as ionization interfaces, such as ESI and APCI were confirmed, approximately, as identical ones. Out of ionization modes, negative ionization was preferred: furnishing higher sensitivity than the corresponding system with positive ionization mode.

(6) In order to define fine structural differences the best methodological strategy proved to be, certainly in the basic research area only, the on-line HPLC–DAD–MS–NMR system.

(7) GC, in combination with mass detection (GC–MS, GC–MS–MS) furnishes excellent resolution.

  (i) Without derivatization identification of aglycones were reported only.

  (ii) Subsequent to derivatization, performing various silylating agents, excellent selectivity and spectacular sensitivity have been achieved.

Acknowledgements

References

[1] B.H. Havsteen, Pharm. Ther. 96 (2002) 67.
[2] P.C.H. Hollman, J. Sci. Food. Agric. 80 (2000) 1081.
[3] H.M. Rawel, S. Rohn, J. Kroll, Int. J. Biol. Maromolecules 32 (2003) 109.
[4] S.A. Aherne, N.M. O'Brien, Nutrition 18 (2002) 75.
[5] M. Netzel, G. Strass, C. Kaul, I. Bitsch, H. Dietrich, R. Bitsch, Food Res. Int. 35 (2002) 213.
[6] K.Y. Park, G.O. Jung, K.T. Lee, J. Choi, M.Y. Choi, G.T. Kim, H.J. Jung, H.J. Park, J. Ethnophar. 90 (2004) 73.
[7] M.V.S. Elipe, Anal. Chim. Acta 497 (2003) 1.
[8] M. Stobiecki, Phytochem. 54 (2000) 237.
[9] G.C. Kite, N.C. Veitch, R.J. Grayer, M.S.J. Simmonds, Biochem. Syst. Ecol. 31 (2003) 813.
[10] M. Careri, A. Mangia, M. Musci, J. Chromatogr. A 794 (1998) 263.
[11] A. Chandra, M.G. Nair, A. Iezzoni, J. Agric. Food. Chem. 40 (1992) 967.
[12] A. Chandra, M.G. Nair, A.F. Iezzoni, J. Agric. Food. Chem. 41 (1993) 1062.
[13] B. Buszewski, S. Kawka, Z. Suprynowicz, T. Wolski, J. Pharm. Biom. Anal. 11 (1993) 211.
[14] K. Ishii, S. Urano, T. Furuta, Y. Kasuya, J. Chromatogr B. 655 (1994) 300.
[15] B. Liu, D.R. Ferry, L.W. Seymour, P.G. deTakáts, D.J. Kerr, J. Chromatogr. B 666 (1995) 149.
[16] K. Ishii, T. Furuta, Y. Kasuya, J. Chromatogr. B 683 (1995) 225.
[17] K. Ishii, T. Furuta, Y. Kasuya, J. Chromatogr. B 704 (1997) 299.
[18] E. Revilla, J.M. Ryan, G. Martin-Ortega, J. Agric. Food. Chem. 46 (1998) 4592.
[19] V. Wyk, B.E. Winter, Biochem. Syst. Ecol. 22 (1994) 813.
[20] C. Ewald, S. Fjelkner-Modig, K. Johansson, I. Sjöholm, B. Akesson, Food Chem. 64 (1999) 231.
[21] C. Manach, O. Texier, C. Morand, V. Crespy, F. Régérat, C. Demigné, C. Rémésy, Free Rad. Med. 27 (1999) 1259.
[22] D. Romanova, D. Grancai, B. Jozova, P. Bozek, A. Vachálková, J. Chromarogr. A 870 (2000) 463.
[23] M.A. Awad, A. de Jager, Posth. Biol. Techn. 20 (2000) 15.
[24] M.A. Awad, A. de Jager, L.M. van Westing, Sci. Hort. 83 (2000) 249.
[25] J.A. Manthey, K. Grohmann, M.A. Berhow, B. Tisserat, Plant Physiol. Biochem. 38 (2000) 333.
[26] M.A. Awad, A. de Jager, L.H.W. van der Plas, A.R. van der Krol, Sci. Hort. 90 (2001) 69.
[27] N.P. Seeram, L.D. Bourquin, M.G. Nair, J. Agric. Food Chem. 49 (2001) 4924.
[28] F.M. Wang, T.W. Yao, S. Zeng, J. Pharm. Biomed. Anal. (2003) 1.

[29] A. Swiderski, P. Muras, H. Koloczek, Sci. Hort. 100 (2004) 139.
[30] A. Rehwald, B. Meier, O. Sticher, J. Cromatogr. A 677 (1994) 25.
[31] W.E. Bronner, G.R. Beecher, J. Chromatogr. A 705 (1995) 247.
[32] P.G. Pietta, C. Gardana, P.L. Mauri, R. Maffei-Facino, M. Carini, J. Chromatogr. B 673 (1995) 75.
[33] F. Kader, B. Rovel, M. Girardin, M. Metche, Food Chem. 55 (1996) 35.
[34] O.M. Palomino, P. Gomez-Serranillos, E. Carretero, A. Villar, J. Chromatogr. A 731 (1996) 103.
[35] A. Crozier, E. Jensen, M.E.J. Lean, M.S. McDonald, J. Chromatogr. A 761 (1997) 315.
[36] M. Keinanen, R. Julkunen-Tiitto, J. Chromatogr. A 793 (1998) 370.
[37] P. Mouly, E.M. Gaydou, A. Auffray, J. Chromatogr. A 800 (1998) 171.
[38] A. Escarpa, M.C. Gonzalez, J. Chromatogr. A 823 (1998) 331.
[39] W.E. Bronner, G.R. Beecher, J. Chromatogr. A 805 (1998) 137.
[40] J.P. Goiffon, P.P. Mouly, E.M. Gaydou, Anal. Chim. Acta 382 (1999) 39.
[41] O. Palomino, M.P. Gómez-Serranillos, K. Slowing, E. Carretero, A. Villar, J. Chromatogr. A 870 (2000) 449.
[42] H. Wang, K. Helliwell, Food Res. Int. 34 (2001) 223.
[43] H. Chen, Y. Zuo, Y. Deng, J. Cromatogr. A 913 (2001) 387.
[44] M. Ye, Y. LI, H. Liu, X. Ji, J. Pharm. Biomed. Anal. 28 (2002) 621.
[45] A. Escarpa, M.D. Morales, M.C. Gonzales, Anal. Chim. Acta 460 (2002) 61.
[46] G. Shui, P. Leong, J. Cromatogr. A 977 (2002) 89.
[47] E. Belajova, M. Suhaj, Food Chem. 86 (2004) 339.
[48] M.V. Martínez-Ortega, M.C. García-Parilla, A.M. Troncoso, Anal. Chim. Acta 502 (2004) 49.
[49] S. de Pascual-Teresa, D. Treutter, J.C. Rivas-Gonzalo, C. Santos-Buelga, J. Agric. Food Chem. 46 (1998) 4209.
[50] Y. Wakui, E. Yanagisawa, E. Ishibashi, Y. Matsuzaki, S. Takeda, H. Sasaki, M. Aburada, T. Oyama, J Chromatogr. 575 (1992) 131.
[51] P. Gamache, E. Ryan, I.N. Acworth, J. Chromatogr. 635 (1993) 143.
[52] M. Careri, L. Elviri, A. Mangia, M. Musci, J. Chromatogr. A 881 (2000) 449.
[53] R. Bugianesi, M. Serafini, F. Simone, D. Wu, S. Meydani, A. Ferro-Luzzi, E. Azzini, G. Maiani, Anal. Chem. 284 (2000) 296.
[54] S.M. Wittemer, M. Veit, J. Chromatogr. B 793 (2003) 375.
[55] D.A. Lewis, W.N. Fields, G.P. Shaw, J. Ethnoph. 65 (1999) 283.
[56] W. Li, J.F. Fitzloff, J. Pharm. Biomed. Anal. 30 (2002) 67.
[57] P.A. Tarantilis, G. Tsoupras, M. Polissiou, J. Chromatogr. A 699 (1995) 107.
[58] B. Ducrey, J.L. Wolfender, A. Marston, K. Hostettmann, Phytochem. 38 (1995) 129.
[59] X. He, L. Lin, L. Lian, J. Chromatogr. A 755 (1996) 127.
[60] X. He, L. Lian, L. Lin, M.W. Bernart, J. Chromatogr. A 791 (1997) 127.
[61] N. Chaves, J.J. Ríos, C. Gutierrez, J.C. Escudero, J.M. Olísa, J. Chromatogr. A 799 (1998) 111.
[62] U. Justesen, P. Knuthsen, T. Leth, J. Chromatogr. A 799 (1998) 101.
[63] M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9.
[64] S. Hakkinen, S. Auriola, J. Chromatogr. A 829 (1998) 91.
[65] J.F. Stevens, A.W. Taylor, M.L. Deinzer, J. Chromatogr. A 832 (1999) 97.
[66] L.Z. Lin, X.G. He, M. Lindenmaier, G. Nolan, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordell, J. Chromatogr. A 876 (2000) 87.
[67] P. Waridel, J.L. Wolfender, K. Ndjoko, K.R. Hobby, H.J. Major, K. Hostettmann, J. Chromatogr. A 926 (2001) 29.
[68] A. Schieber, P. Keller, R. Carle, J. Chromatogr. A 910 (2001) 265.
[69] C.W. Huck, M.R. Buchmeiser, G.K. Bonn, J. Chromatogr. A 943 (2001) 33.

[70] R.J. Hughes, T.R. Croley, C.D. Metcalfe, R.E. March, J. Mass Spectrom. 210-211 (2001) 371.
[71] N. Fabre, I. Rustan, E. de Hoffman, J. Quetin-Leclercq, Am. Soc. Mass Spectrom. 12 (2001) 707.
[72] M. Carini, G. Aldini, S. Furlanetto, R. Stefani, R.M. Facino, J. Pharm. Biomed. Anal. 24 (2001) 517.
[73] T.P.J. Mulder, C.J. van Platerink, P.J.W. Schily, J.M.M. van, Amelsvoort, J. Chromatogr. B 760 (2001) 271.
[74] J. Qu, Y. Wang, G. Luo, Z. Wu, J. Chromatogr. A 928 (2001) 155.
[75] B. Zywicki, T. Reemtsma, M. Jekel, J. Chromatogr. A 970 (2002) 191.
[76] F. Sanches-Rabaneda, O. Jauregiu, R.M. Lamuela-Raventós, J. Bastida, F. Vilodomat, C. Codina, J. Chromatogr. A 1008 (2003) 57.
[77] S.M. Boué, C.H. Carter-Wientjes, B.Y. Shih, T.E. Cleveland, J. Chromatogr. A 991 (2003) 61.
[78] V. Carbone, P. Montoro, N. de Tomassi, C. Pizza, J. Pharm. Biomed. Anal. 34 (2003) 295.
[79] E. de Rijke, H. Zappey, F. Ariese, C. Gooijer, U.A.Th. Brinkman, J. Chromatogr. A 984 (2003) 45.
[80] F.D.P. Andrade, L.C. Santos, M. Datchler, K. Albert, W. Vilegas, J. Cromatogr. A 953 (2002) 287.
[81] A. Ogawa, H. Arai, H. Tanizawa, T. Miyahara, T. Toyooka, Anal. Chim Acta 383 (1999) 221.
[82] A. Rehwald, B. Meier, O. Sticher, Pharm. Acta Helv. 69 (1994) 153.
[83] H. Wang, M.G. Nair, A.F. Iezzoni, G.M. Strasburg, A.M. Booren, J.I. Gray, J. Agric. Food. Chem. 45 (1997) 2556.
[84] W.B. Phippen, J.E. Simon, J. Agric. Food. Chem. 46 (1998) 1734.
[85] T. Fossen, A.T. Pedersen, Q.M. Andersen, Phytochem. 47 (1998) 281.
[86] T. Fossen, A. Larsen, B.T. Kiremire, Q.M. Andersen, Phytochem. 51 (1999) 1133.
[87] H. Wang, M.G. Nair, G.M. Strasburg, A.M. Booren, J.I. Gray, J. Agric. Food Chem. 47 (1999) 840.
[88] Zs. Kovács, Z. Dinya, Microchem J. 67 (2000) 57.
[89] E.L. Johnson, W.F. Schmidt, D. Cooper, Plant. Physiol. Biochem. 40 (2002) 89.
[90] Ph. Morin, F. Villard, M. Dreux, J. Chromatogr. 628 (1993) 161.
[91] B.L. Lee, C.N. Ong, J. Chromatogr. A 881 (2000) 439.
[92] C.T. da Costa, D. Horton, S.A. Margolis, J. Chromatogr. A 881 (2000) 403.
[93] C. Bjergegaard, S. Michaelsen, K. Mortensen, H. Sorensen, J. Chromatogr. 652 (1993) 477.
[94] Ph. Morin, F. Villard, M. Dreux, J. Chromatogr. 628 (1993) 153.
[95] F. Ferreres, M.A. Blazquez, M.I. Gil, F.A. Tomás-Barberán, J. Chromatogr. A 669 (1994) 268.
[96] K.L. Li, S.J. Sheu, Anal. Chim. Acta 313 (1995) 113.
[97] P.J. Larger, A.D. Jones, C. Dacombe, J. Chromatogr. A 799 (1998) 309.
[98] M.A. Rodriguez-Delgado, M.L. Pérez, R. Corbella, G. Gonzalez, F.J. Garcia Montelongo, J. Chromatogr. A 871 (2000) 427.
[99] G.D. Monache, R. Scurria, A. Vitali, B. Botta, B. Monacelli, G. Pasqua, C. Palocci, E. Cernia, Phytochemistry. 37 (1994) 893.
[100] T.M. Upson, R.J. Grayer, J.R. Greenham, C.A. Williams, F. Al-Ghamdi, F.H. Chen, Biochem. Syst. Ecol-ogy 28 (2000) 991.
[101] J. Onyilagha, A. Bala, R. Hallett, M. Gruber, J. Soroka, N. Westcott, Biochem. Syst. Ecol. 31 (2003) 1309.
[102] T. Kartnig, I. Göbel, J. Chromatogr. A 740 (1996) 99.
[103] E. de Rijke, H.C. Joshi, H.R. Sandersen, F. Ariese, U.A.Th. Brinkman, C. Gooijer, Anal. Chim. Acta 468 (2002) 3.
[104] H.R. Shulten, Anal. Chem. 61 (1989) 221.
[105] R. Christov, V. Bankova, J. Chromatogr. 623 (1992) 182.
[106] T.J. Shmidt, I. Merfort, J. Chromatogr. 634 (1993) 350.
[107] T.J. Schmidt, I. Merfort, G. Willuhm, J. Chromatogr. A 669 (1994) 236.
[108] M.D. Gillén, M.J. Manzanos, Food Chem. 63 (1998) 373.

[109] M.C. Lin, M.J. Tsai, K.C. Wen, J. Chromatogr. A 830 (1999) 387.

[110] T.J. Schmidt, G. Willuhn, Biochem. Syst. Ecol. 28 (2000) 133.

[111] M.P. Fernandez, P.A. Watson, C. Breuil, J. Chromatogr. A 922 (2001) 225.

[112] R.D. Shmid, R. Mues, J.H. McReynolds, G.V. Velde, N. Nakatani, E. Rodrígez, T.J. Mabry, Phytochem. 12 (1973) 2765.

[113] K.R. Markham, K.A. Mitchell, A.L. Wilkins, J.A. Daldy, Y. Lu, Phytochem. 42 (1996) 205.

[114] T. Katagi, A. Horri, Y. Oomura, H. Miyakawa, T. Kyu, Y. Ikeda, K. Isoi, J. Chromatogr. 79 (1973) 45.

[115] E. Bombardelli, A. Bonati, B. Gabetta, E.M. Martinelli, G. Mustich, J. Chromatogr. 139 (1977) 111.

[116] A. Baj, E. Bombardelli, B. Gabetta, E.M. Martinelli, J. Chromatogr. 279 (1983) 365.

[117] W. Greenaway, T. Scaysbrook, F.R. Whatley, Phytochem. 27 (1988) 3513.

[118] W. Greenaway, F.R. Whatley, Phytochem. 29 (1990) 2551.

[119] S. English, W. Greenaway, F.R. Whatley, Phytochem. 30 (1991) 531.

[120] W. Greenaway, I. Gümüsdere, F.R. Whatley, Phytochem. 30 (1991) 1883.

[121] W. Greenaway, F.R. Whatley, Phytochem. 30 (1991) 1887.

[122] W. Greenaway, S. English, J. May, F.R. Whatley, Biochem. Syst. Ecol. 19 (1991) 507.

[123] V. Bankova, R. Christov, G. Stoev, S. Popov, J. Chromatogr. 607 (1992) 150.

[124] J.F. Stevens, H. Hart, E.T. Elema, A. Bolck, Phytochem. 41 (1996) 503.

[125] D.G. Watson, E.J. Oliveira, J. Chromatogr. B 723 (1999) 203.

[126] G.J. Soleas, J. Yan, D.M. Goldberg, J. Chromatogr. B 757 (2001) 161.

[127] F. Deng, S.W. Zito, J. Chromatogr. A 986 (2003) 121.

[128] E. Prytzyk, A.P. Dantas, K. Salamo, A.S. Pereira, V.S. Bankova, S.L. DeCastro, F.R.A. Neto, J. Ethnopharm. 88 (2003) 189.

[129] S. Scalia, L. Giuffreda, P. Pallado, J. Pharm. Biomed. Anal. 21 (1999) 549.

[130] Z. Piñeiro, M. Palma, C.G. Barroso, J. Chromatogr. A 1026 (2004) 19.

[131] M.V. Martinez-Ortega, M.C. Garcia-Parilla, A.M. Troncoso, Anal. Chim. Acta 502 (2004) 49.

[132] M.G.L. Hertog, P.C.H. Holmann, D.P. Venema, J. Agric. Food Chem. 40 (1992) 1591.

[133] I. Molnár-Perl, Zs. Füzfai, Chromatographia 60 (2004) S145–S153.