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Determination of selected phytochemicals by reversed-phase high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection

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Abstract

A robust, routinely manageable and sensitive RP-HPLC method combined with UV (270 nm) and ESI-MS detection was established for the determination of abundant pertinent phenolic compounds (phytochemicals) from various biological matrices. Phytochemicals were extracted by aqueous methanol (80%), extracts were analysed without further purification. Baseline separation was achieved within 30 min for 19 phytochemicals and excellent sensitivity (6–42 pmol at $S/N=3$) was obtained. The identity of the phytochemicals was confirmed with standard compounds and with LC–MS. The repeatabilities for the majority of the phytochemicals ranged between 3% and 6%. The practicability of the method was shown in complex biological matrices by analysing onion and soybean extracts. This generally applicable technique may serve as a valuable tool for a rapid screening and a specific measurement of phytochemicals in food extracts and biological fluids and serve as analytical instrument for future biochemical and physiological studies.

Keywords: Phytochemicals; Phenolic compounds

1. Introduction

At present naturally occurring phytochemicals are of major scientific interest. Technically, the term ''phytochemical'' refers to every naturally occurring chemical substance present in plants, especially to those phytochemicals that are biologically active [1]. They occur in small amounts in all higher plants and in all parts of plants – wood, bark, stems, pods, leaves, fruits, roots, flowers, pollen and seeds [2,3]. Major phytochemicals are phenolic acids, such as cinnamic or ferulic acid, flavones, isoflavones, flavonols, flavanes, catechins, anthocyanidins and many other polyphenols. During the last decade data from both experimental and epidemiological studies have been accumulated showing that phytochemicals possess potential chemopreventive properties [3–5].

Considering this background it is surprising that until now little attention has been paid to quantitative aspects of the analysis of phytochemicals in food and biological fluids. Early findings were mainly obtained with paper chromatography, thin-layer chromatography and spectrophotometry [6,7]. More recently multitudinous application of high-performance liquid chromatography (HPLC) has been recognised as a powerful tool in analyses of single phyto-

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chemicals. In these reports adequate separation and quantification of selected subclasses of phenolic compounds like flavonoids, stilbenes and phenolic acids, etc. have been performed in certain foods like grains [8], vegetables and fruits [9–12], tea [9,13–15], wine [9,16,17] and honey [18]. Although these studies are well reflecting an appropriate implication of HPLC in food chemistry they are mostly carried out for taxonomic purposes. Thus, only major subclasses were identified and little attention has been paid to a verified screening approach dealing with different groups of phenolic compounds, especially those of flavonoids, present in complex matrices.

In this paper we present a newly developed, generally applicable HPLC method for the determination of 19 abundant phenolic compounds. This method may be of value in future studies, in which the physiological and biochemical action of phytochemicals will be investigated.

2. Materials and methods

2.1. Apparatus for HPLC

2.1.1. Gradient system with UV detection

The HPLC system (Sykam, Gilching, Germany) consisted of a solvent delivery system S 1000, a HPLC controller S 2000, a low-pressure gradient mixer S 8110, and a Rheodyne injection valve 7125 with a 20-μl filling loop. UV absorbance was monitored with a 655 A-22 variable-wavelength monitor (Merck–Hitachi) at 270 nm with a flow cell of 11 μl (5 mm). Continuous on-line quantitation of the HPLC results was obtained with a Chromatopac C-R6A data processor (Shimadzu).

2.1.2. Gradient system with mass spectrometric detection

Unknown compounds were identified with a gradient HP HPLC system series 1100 (Hewlett-Packard, Böblingen, Germany) combined with a mass spectrometric detector with atmospheric pressure ionisation in the electrospray ionisation (ESI) mode (Micro Mass Platform II, Mass Lynx 4.0, Manchester, UK). Source temperature was maintained at 120°C, cone voltage at −40 eV and acceleration lens potential at 0.5 kV. Negative ion characterisation was performed in the m/z range of 100–800 at a scan rate of 0.5 scans/s, and a multiplier voltage of 650 V.

2.2. Reagents and standards

Ultrapure water was generated with a Elga MAXIMA water purification system, including reverse osmosis, activated carbon and ion-exchange cartridges (Elga, Lane End, UK). The chemicals used were of analytical grade and the solvents of HPLC-grade. Formononetin was synthesised at the Institute for Biological Chemistry and Nutrition [its structure was confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS)]. Catechin, epicatechin, flavone, myricetin, naringenin, daidzein, genistein, morin and resveratrol were purchased from Aldrich (Steinheim, Germany); apigenin, kaempferol, naringin, quercetin and rutin from Fluka (Buchs, Switzerland); malvin chloride was obtained from Roth (Karlsruhe, Germany); biochanin A, daidzin and genistin from Extrasynthese (Genay, France).

2.3. HPLC conditions

Gradients were formed between two helium degassed solvents. Solvent A was acetonitrile–water–formic acid (10:90:5, v/v/v) and solvent B was composed of acetonitrile–water–formic acid (90:10:5, v/v/v). Gradient conditions: 0–3 min 0% B; 3–4 min 0–17% B; 4–22 min 17–28% B; 22–23 min 28–50% B; 23–29.5 min 50% B.

A 125 mm×4.6 mm I.D. Hypersil 120 octadecyl silica (particle size 3 μm) column was used; it was obtained from Muder and Wochele (Berlin, Germany). The column was at room temperature, with a flow-rate of 0.9 ml/min. Injection volume was 20 μl.

2.4. Biological applications

The practicability of the method in complex biological matrices was demonstrated by analysing onion and soybean extracts, without and with hydrolyses.

Briefly, 1 g of onion was homogenised and subsequently extracted with 8 ml methanol–water (8:2, v/v) (containing 7.2 mg/l naringin as internal

standard for verifying the completeness of the extraction procedure) by tumbling for 2 h at room temperature. The mixture was centrifuged at 4000 rpm for 20 min at 4°C. The supernatant was analysed with HPLC without further purification. Peak area of quercetin was corrected by the peak area of the added internal standard naringin and compared to the area of a known quercetin standard in order to estimate the onion's quercetin concentration.

Soybeans were ground in an electric coffee mill. A powdered portion of 500 mg soybean was defatted by hexane extraction (2×10 ml and subsequent centrifugation). The pellet was extracted with 8 ml methanol–water (8:2, v/v) (containing 7.2 mg/l naringin as internal standard) and centrifuged at 4000 rpm for 20 min. The extract was analysed with HPLC without further purification.

Recovery was assessed by adding 59.1 nmol (20.0 μg) of quercetin to 1.00 g of homogenised onion and 62.5 nmol (26.0 ng) daidzin, 73.3 nmol (31.7 ng) genistin, 31.9 nmol (8.1 ng) daidzein and 91.4 nmol (24.7 ng) genistein, respectively to 50 mg powdered soybean (three additions each) and analysed as a biological sample.

In order to transform glycosides to aglycons, acid hydrolysis of glycosidic compounds was performed with 2 mol/l HCl (2 ml extract+2 ml 4 mol/l HCl) for 2 h at 130°C in sealed vessels. The hydrolysed solution was neutralised with 4 mol/l NaOH and adjusted to 10 ml.

3. Results

3.1. Standard method

Using the conditions specified under materials and methods, the system enables separation of 19 selected phytochemicals within 30 min. The retention times, limits of detection (LODs), repeatabilities and variables for the calibration curves are given in Table 1. Peak assignments were made with single compound injections. Baseline separation was successful-

Table 1
Retention times (t_R), limits of detection (LODs; $S/N=3$), calibration curve ($ax+b$; correlation coefficient, r, area·10^3, concentration in μmol/l), repeatabilities (RSD) and standard concentrations (c) for determining the repeatabilities of the phytochemicals (UV detection, 270 nm; unless indicated, 10 replicate determinations)

	t_R±SD (min)	LOD (pmol)	a	b	r	RSD	c (μmol/l)
Catechin	3.28±0.08	40	1.56	−0.90	0.9909	5.4	19.4
Epicatechin	5.57±0.16	40	2.10	0.60	0.9977[a]	11.5	23.0
Malvin chloride	7.03±0.06	40	5.45	−0.10	0.9964	4.7	20.5
Daidzin	7.28±0.06	6	10.18	1.41	0.9977[a]	5.7	15.2
Rutin	8.08±0.15	8	9.21	2.78	0.9984[a]	4.5	22.6
Genistin	8.53±0.15	6	11.39	3.36	0.9971[a]	5.1	17.6
Naringin	8.88±0.21	8	6.87	0.00	0.9999[a]	3.1	22.7
Myricetin	9.87±0.13	13	5.06	−1.96	0.9953	7.2	6.1
Resveratrol	10.81±0.13	16	4.59	0.14	0.9979[b]	4.2	20.5
Morin	11.19±0.12	42	5.48	−14.89	0.9914	5.1	17.9
Daidzein	12.10±0.12	6	11.50	−0.39	0.9985[b]	4.4	8.0
Quercetin	13.14±0.12	16	7.30	−6.75	0.9963[b]	4.4	20.2
Naringenin	15.25±0.14	8	4.97	0.58	0.9964	4.8	22.7
Genistein	16.46±0.14	8	13.36	−6.24	0.9987[b]	4.1	22.2
Apigenin	17.55±0.16	16	8.19	−2.66	0.9968	6.2	18.6
Kaempferol	17.87±0.16	37	8.88	−38.2	0.9972[c]	8.2	18.6
Formononetin	22.50±0.18	8	10.19	−0.60	0.9980[a]	6.0	21.6
Biochanin A	27.54±0.08	8	15.72	−5.75	0.9968[b]	5.8	19.6
Flavone	28.18±0.09	16	5.83	1.69	0.9977[c]	3.9	21.4

[a] $n=12$.
[b] $n=11$.
[c] $n=9$.

ly achieved for all compounds except for the critical pair apigenin–kaempferol.

The calibration curves were calculated from the LOD to concentrations of 40 μmol/l for each standard compound. The detection limits ranged from 6 pmol per injection for daidzein, daidzin and genistin to 42 pmol per injection for morin ($S/N=3$). Repeatabilities were calculated by ten repeated measurements of the same standard solution (standard concentrations given in Table 1). For the majority of the phytochemicals they ranged between 3% and 6%, myricetin exhibited 7.2%, kaempferol 8.2% and epicatechin 11.5%.

Table 2
Ions in the ESI mass spectra used for verification of compounds from onion and soybean extracts, respectively

	$[M-H]^-$	$[M-glc-H]^-$
Onion		
Quercetin glucoside	463	301
Quercetin diglucoside	625	463
Isorhamnetin glucoside	477	456
Quercetin	301	
Epicatechin	289	
Soybean		
Daidzin	415	253
Genistin	431	269
Daidzein	253	
Genistein	269	

3.2. Onion extract

The UV chromatograms of the non-hydrolysed and hydrolysed onion extract are shown in Fig. 1. Identification of quercetin in the untreated and the hydrolysed onion extract was made by using a quercetin standard solution, which showed a peak at the same retention time.

For quantification we used UV detection. The quercetin content with external standard method yielded 21.8 mg/kg fresh mass and with the internal standard method we yielded a content of 19.5 mg/kg. After acid hydrolysis the main phytochemical in the extract was quercetin, amounting 90.2 mg/kg with the external standard. Recovery of quercetin from onion was shown to be 104.3±5.6%.

The chromatogram of the onion extract (Fig. 1A) showed some peaks with lower retention times than quercetin. Characteristic ions in the LC-ESI mass spectra (Table 2) enabled peak assignment for these compounds to known compounds from onion for which we had no standards. The major phenolic

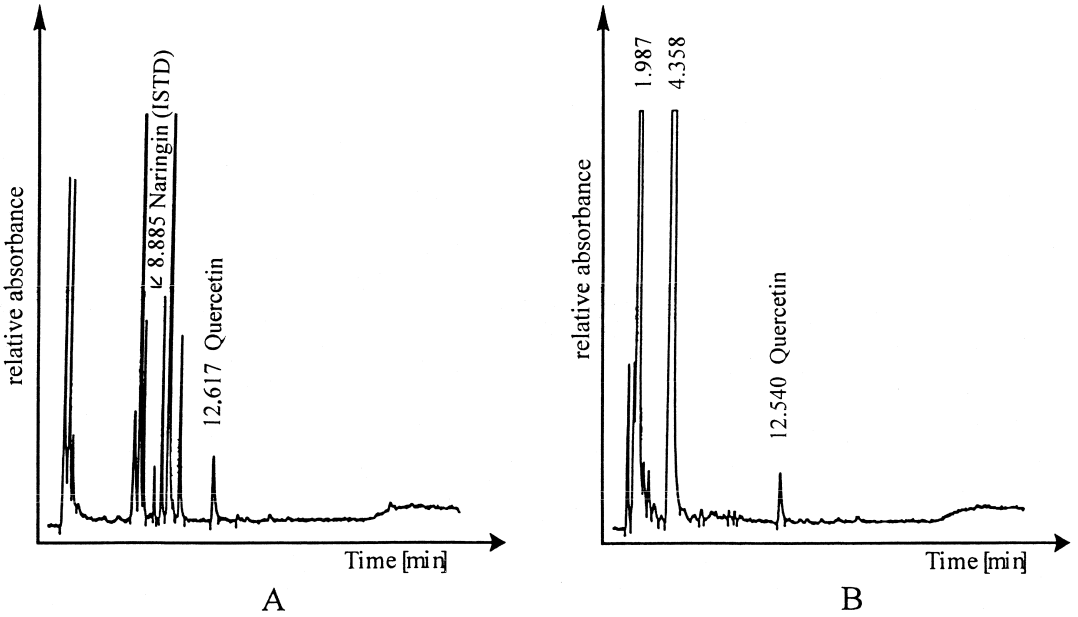

Fig. 1. HPLC analysis of an onion extract without (A) and with acid hydrolysis (B, five-fold diluted).

component was quercetin glucoside. The deprotonated molecular ion $[M–H]^-$ was observed as base peak at m/e 463, whereas the aglycon fragment $[M–glc-H]^-$ was observed at m/e 301. Another compound of the extract was quercetin diglucoside, showing the $[M–H]^-$ at m/e 625 and the $[M–glc-H]^-$ at m/e 463. A further major component was isorhamnetin glucoside identified by the molecular ion $[M–H]^-$ observed at m/e 477 and the fragment ion $[M–glc-H]^-$ at m/e 456. Identification of quercetin was achieved by its characteristic retention time and the molecular ion $[M–H]^-$ at m/e 301. Selected ion monitoring enabled one to additionally prove the presence of epicatechin ($[M–H]^-$ m/e 289) in the onion extract, which however could not be confirmed in the UV chromatogram due to low concentration and matrix signals. In the hydrolysed onion extract (Fig. 1B) two further peaks at t_R 1.987 and t_R 4.358 were observed. Both might indicate phenolic acids, which however could not be identified because of lacking standard compounds and unsuccessful LC–MS identification.

Fig. 2 shows the LC–MS chromatogram of the onion extract with selected ion monitoring for epicatechin (m/e 289), quercetin (m/e 301), quercetin monoglucosid (m/e 463), quercetin diglucosid (m/e 625) and isorhamnetin 4′-glucoside (m/e 477). Differences in retention times between HPLC–UV and HPLC–MS chromatograms can be explained by variations due to different HPLC systems used (different pre-column dead volume, column temperature), but did not affect separation efficacy.

3.3. Soybean extract

In Fig. 3, the UV chromatograms of the soybean extract, non-hydrolysed and hydrolysed are depicted. Identification was achieved by using standard compounds and their retention times for for daidzin, genistin, daidzein and genistein in untreated and partially hydrolised soybean extract, respectively. Differences in retention times can be explained by diurnal temperature variations in summer (no column oven). Identity was verified by addition of standard substances.

UV detection was used for the quantification of isoflavones content in the non-hydrolysed soybean extract. The content of the major isoflavones daidzin, genistin, daidzein and genistein yielded 670 mg/kg, 602 mg/kg, 100 mg/kg, 144 mg/kg, respectively with external standard.

Internal standard calculation for daidzin, genistin, daidzein and genistein revealed 692 mg/kg, 622 mg/kg, 103 mg/kg, 148 mg/kg, respectively. Recoveries of daidzin, genistin, daidzein and genistein from soybean were $82.1\pm1.4\%$, $88.0\pm1.3\%$, $103.1\pm2.3\%$, $104.6\pm3.7\%$, respectively. Identity of the compounds was confirmed by characteristic ions in their ESI mass spectra (Table 2). Two peaks in the hydrolysed soybean extract (t_R 2.335 and t_R 5.712, Fig. 3B) might indicate phenolic acids, which however could not be identified because of lacking standard compounds and unsuccessful LC–MS identification.

4. Discussion

In this study, a rapid and convenient assay based on reversed-phase HPLC combined with UV and mass spectrometric detection, respectively, was developed. The method permits rapid specific and sensitive measurement of phenolic phytochemicals in standard solution as well as in complex biological material. The detection limits for UV detection found in the present work were comparable to those described in previous reports [18,19]. This technique may serve as a generally applicable tool for a rapid screening of all kind of biological matrices and quantification of the selected phytochemicals.

The validity and practicability of the method was exemplified by analysing onion and soybean. Onion has a simple flavonol composition that is dominated by two components: quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside, which constitute over 85% of the total flavonol fraction in onion. The remaining 15% is composed of quercetin, quercetin 3-monoglucoside and isorhamnetin 3-monoglucoside [20]. Indeed, along with the quercetin peak some unknown peaks were monitored in the UV chromatogram (Fig. 1). Without reference substances conclusive assignments of quercetin monoglucoside, quercetin diglucoside and isorhamnetin 4′-glucoside were not possible with UV detection, while, as suggested earlier [21], LC–MS is a suitable tool for their identification. In this previous work isorhamnetin

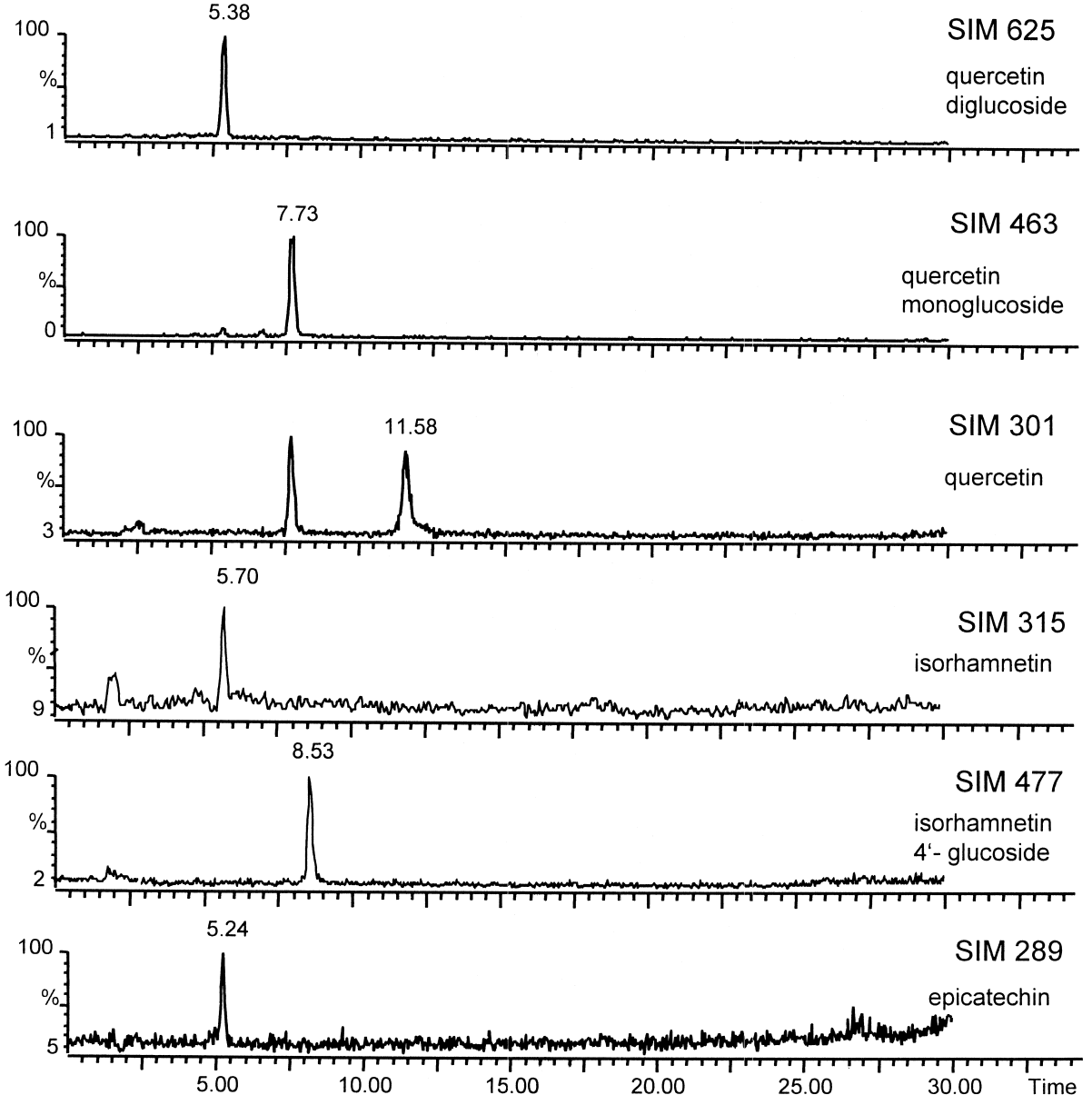

Fig. 2. Identification of signals from an onion extract by single ion monitoring (SIM).

was additionally identified. Despite using single ion monitoring, we were unable to detect this compound. On the other hand we detected epicatechin by using MS detection (SIM mode), but not with UV detection.

The quercetin content of onion shows great variations, probably due to analytical methods, seasonal variation, cultivar, storage and sampling conditions. Free quercetin concentrations in onion were reported to yield 16.3 mg/kg and 6.0 mg/kg fresh mass, dependent on cultivar [22], in good agreement with contents of about 20 mg/kg found in the present

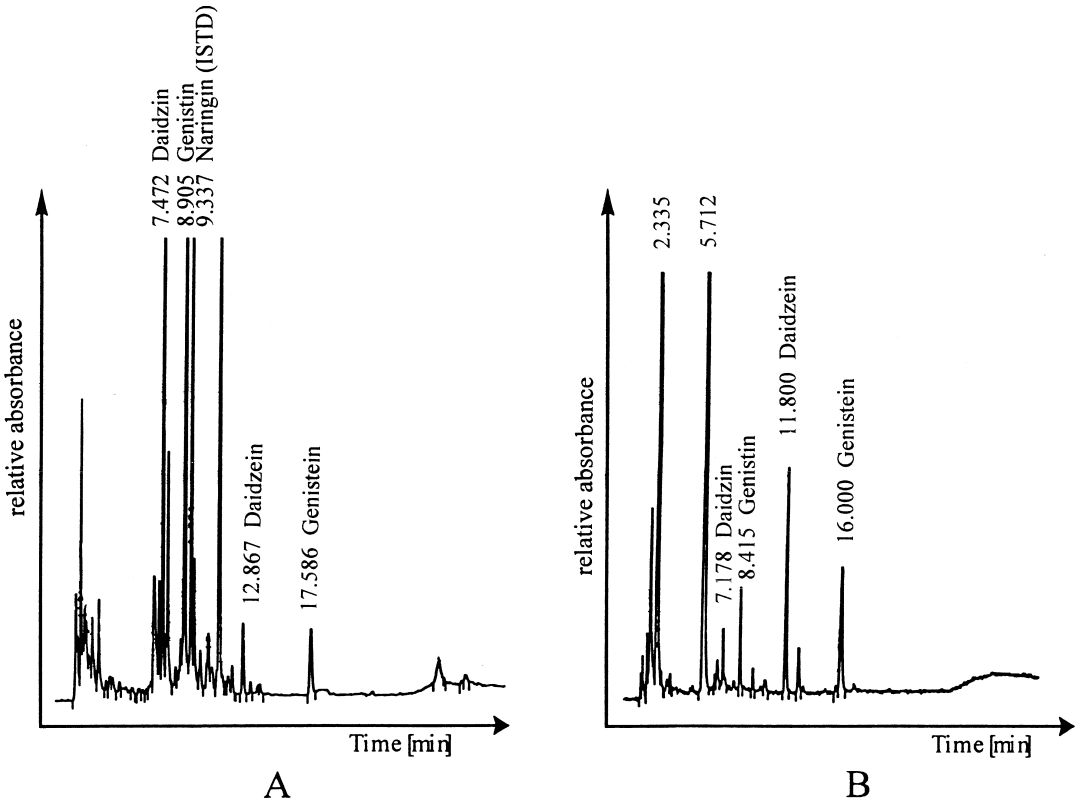

Fig. 3. HPLC analysis of a soybean extract without (A, three-fold diluted) and with acid hydrolysis (B, five-fold diluted).

study. Indeed, the recovery of quercetin was good (104%). Higher contents (15–62 mg/kg fresh mass) were reported by Bilyk et al. [23].

Quercetin is claimed to be the major phytochemical in acid hydrolised onion extracts (544 mg/kg fresh mass [24]). However, in the present study we found only 90 mg/kg fresh mass.

The daidzin, genistin, daidzein and genistein con-

Table 3
Phytochemicals in different matrices

Matrix	Phytochemicals	Ref.
Onion	Quercetin glucoside, quercetin diglucoside, isorhamnetin glucoside, quercetin	[21]
Soy	Daidzin, genistin, daidzein, genistein	[19]
Wine	Catechin, epicatechin, rutin, resveratrol, quercetin	[26]
Tea (green, black)	Quercetin, kaempferol, myricetin	[10]
Celery	Apigenin, luteolin	[27]
Citrus	Naringin, hesperidin, neohesperidin, nobiletin, tangeretin	[28]
Buckwheat	Quercetin, rutin, hyperin	[29]

tent in soybean were consistent with previous reports [19,25] and in the present study their identity was confirmed by their retention times and characteristic ions in their LC-ESI mass spectra. Several unknown peaks in the soybean extract with low retention times represent presumably signals from malonyl glucosid conjugates of daidzein and genistein (Fig. 2). Hydrolytic cleavage decreased peak height of these early eluting peaks, in fact yielding high amounts of genistein and daidzein.

The newly established method is routinely manageable and allows quantification of major phenolic components in different biological materials (Table 3) with UV detection and identification of other phytochemicals with ESI-MS detection. At present this fascinating topic is mainly discussed in terms of epidemiology and taxonomy while related physiology and biochemistry are warranted. Indeed the present method may facilitate such future studies.

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