Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 549–556

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Simultaneous determination of phenolic acids and flavonoids in *Lycium barbarum* Linnaeus by HPLC–DAD–ESI-MS

B. Stephen Inbaraj [a], H. Lu [a], T.H. Kao [a], B.H. Chen [a,b,*]

[a] *Department of Food Science, Fu Jen University, Taipei 242, Taiwan*
[b] *Graduate Institute of Medicine, Fu Jen University, Taipei 242, Taiwan*

ARTICLE INFO

Article history:
Received 1 July 2009
Received in revised form 31 August 2009
Accepted 5 September 2009
Available online 11 September 2009

Keywords:
Lycium barbarum Linnaeus
Chinese herb
Phenolic acid
Flavonoid
HPLC–DAD–ESI-MS

ABSTRACT

A high-performance liquid chromatography–diode array detection–mass spectrometry method with electrospray ionization mode (HPLC–DAD–ESI-MS) was developed for simultaneous determination of phenolic acids and flavonoids in fruits of *Lycium barbarum* Linnaeus, a widely used traditional Chinese herb possessing vital biological activity. Both phenolic acids and flavonoids were extracted with 50% ethanol and purified using a polymeric solid phase extraction cartridge followed by HPLC–DAD–ESI-MS analysis. By employing a Vydac C18 column, a total of 52 phenolic acids and flavonoids were separated within 70 min using a gradient mobile phase of 0.5% (v/v) formic acid in water and acetonitrile–water (94:6, v/v) with flow rate at 1 mL/min, column temperature at 30 °C and detection wavelength at 280 nm. Of 52 compounds, 15 phenolic acids and flavonoids were positively identified based on both absorption and mass spectra, with the remaining 37 tentatively identified by comparison of absorption spectra with reported values in the literature. Internal standards 3-hydroxybenzoic acid and hesperidin were used for quantitation of phenolic acids and flavonoids, respectively. Among the 15 positively identified compounds, quercetin-rhamno-di-hexoside was present in largest mass fraction (438.6 μg/g), followed by quercetin-3-O-rutinoside (281.3 μg/g), dicaffeoylquinic acid isomers (250.1 μg/g), chlorogenic acid (237.0 μg/g), quercetin-di-(rhamnohexoside) (117.5 μg/g), quercetin-di-(rhamno)-hexoside (116.8 μg/g), kaempferol-3-O-rutinoside (97.7 μg/g), isorhamnetin-3-O-rutinoside (72.1 μg/g), p-coumaric acid (64.0 μg/g), caffeic acid (23.7 μg/g) and vanillic acid (22.8 μg/g).

1. Introduction

Phenolic compounds constitute a large group of secondary plant metabolites. Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids and the most common among them are caffeic acid, p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, vanillic acid and procatechuic acid, which frequently occur in fruits and vegetables as esters or glycosides. In recent years, phenolic acids have received considerable attention because of their protective role against cancer and heart diseases. This may be attributed to their antioxidant activity which was reported to be higher than the vitamin antioxidants [1–3]. On the other hand, flavonoids belong to a family of C_6–C_3–C_6 polyphenol compounds and the major flavonoid subclasses in the diet include flavonol, flavone, flavanone, flavanol, anthocyanin and isoflavone. Of the various flavonols, quercetin is the most frequently studied and has been shown to possess anti-inflammatory and anticancer activities [4–6]. In view of the impact of both phenolic acids and flavonoids on human health, it is vital to learn about their amounts and varieties in medicinal plants.

The extraction of phenolic acids or flavonoids is often carried out by polar solvents like water, ethanol, methanol or a mixture of water and ethanol or methanol [7,8]. Qian et al. [7] compared the extraction efficiency of flavonoids in *Lycium Chinese* Mill. fruits with 100% water, 50% ethanol and 90% ethanol, with a high yield (1497 ± 70 mg/kg) being attained by using 95% ethanol. In another study, the extraction efficiency of phenolic acids in propolis was evaluated using four solvent systems, 70% ethanol, 90% ethanol, 100% acetone, and hexane plus acetone, and acetone was reported to show high extraction efficiency (81% of native propolis) [9]. A solvent system of 90% methanol was employed for the extraction of polyphenols in plant foods, and a recovery ranging from 68 to 92% was reported [8]. In a review article, Molnár-Perl and Füzfai [10] concluded that the optimum extraction conditions for flavonoids in plants can be dependent on the nature of compounds and the matrix from which they are isolated. The separation of phenolic acids and flavonoids has been previously achieved by thin-layer chromatography (TLC) or paper chromatography (PC) [11,12], however, these methods are either

* Corresponding author at: Department of Food Science, Fu Jen University, Taipei 242, Taiwan. Tel.: +886 2 29053626; fax: +886 2 29021215.
E-mail address: 002622@mail.fju.edu.tw (B.H. Chen).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.09.006

lengthy or the separation power is inadequate. An excellent resolution could be achieved by gas chromatography in combination with mass spectrometry (GC–MS) [8,13,14], but the derivatization step makes this method time-consuming. Some other techniques like capillary zone electrophoresis (CZE) and high-performance liquid chromatography–mass spectrometry (HPLC–MS) were demonstrated to provide convincing and satisfactory results [10,15]. The former method (CZE) permits the use of a small amount of solvent, yet it provides lower resolution of complex samples and sensitivity than that could be achieved with LC–MS [10,16]. The negative ionization mode for LC–MS detection was reported to be superior to the positive ionization mode in terms of sensitivity [10,17].

The fruits of *Lycium barbarum* Linnaeus, a traditional Chinese medicinal herb, have been widely used as a major functional component in health foods since numerous studies have demonstrated that it may exhibit vital biological activities such as prevention of age-related macular degeneration, inhibition of cancer cell proliferation and enhancement of immune response [18,19]. This beneficial effect has been attributed to the presence of various functional components including polysaccharides, flavonoids, phenolic acids and carotenoids. In a recent study, we reported a HPLC–DAD–MS method with atmospheric pressure chemical ionization (APCI) mode for determination of 11 free carotenoids and 7 carotenoid esters in *L. barbarum* [20]. However, the amount and variety of phenolic acids and flavonoids in *L. barbarum* still remain uncertain. Therefore, the objectives of this study were to develop a HPLC–DAD–ESI-MS method for qualitative and quantitative determination of phenolic acids and flavonoids in fruits of *L. barbarum*.

2. Materials and methods

2.1. Materials

Phenolic acid standards, including caffeic acid, chlorogenic acid, vanillic acid, p-coumaric acid, ferulic acid, sinapic acid and the internal standard (IS) 3-hydroxybenzoic acid with a purity of 99, 95, 97, 98, 99, 98 and 99%, respectively, were obtained from Sigma (St. Louis, MO, USA). Flavonoid standards, including rutin, taxifolin, luteolin, quercetin, naringenin, apigenin, hesperetin, kaempferol, isorhamnetin and the IS hesperidin with a purity of 95, 85, 98, 98, 95, 95, 95, 90, 95 and 80%, respectively, were also purchased from Sigma, while kaempferol-3-O-rutinoside was from Chromadex (Santa Ana, CA, USA). The HPLC-grade solvents methanol and acetonitrile were procured from LAB-SCAN (Dubin, Ireland) and ethanol (95%) used for extraction was from Taiwan Tobacco and Liquor Co. (Tainan, Taiwan). Formic acid and hydrochloric acid were from Riedel-de-Haën Co. (Seelze, Germany). Deionized water was made using a Milli-Q purification system (Millipore, Bedford, MA, USA). The solid phase extraction (SPE) cartridge Strata-X (200 mg/6 mL, 33 μm, polymeric reversed phase) were from Phenomenex (Torrance, CA, USA). A Vydac 201TP54 C18 column (250 mm × 4.6 mm I.D., 5 μm particle size) from Grace Davison Discovery Science (Deerfield, IL) was used for separation.

2.2. Instrumentation

An 1100 series Agilent HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) composed of a G1312A pump, a G1311A quaternary pump, a G1379A online degasser, a G1316A column temperature controller and a G1315B diode array detector (DAD) was used for separation and quantitation. For identification, the LC–MS–MS analysis was performed on a TSQ Ultra triple quadrupole MS system (Thermo Electron Corporation, MA, USA) equipped with Surveyor Plus LC pump (Model 68649), Surveyor Plus Autosampler (model 76598) and an ESI interface. Data acquisition was performed on a Xcalibur software system. The high-speed centrifuge (model 5810) was from Eppendorf (Hamburg, Germany). The rotary evaporator (model N-1) was from Eyela (Tokyo, Japan). The shaker (V-U) was from Hsiang-Tai Co. (Taipei, Taiwan) and the pH meter (SP-701) from San-Tai Co. (Taipei, Taiwan).

2.3. Extraction and purification of phenolic acids and flavonoids

A method based on Qian et al. [7] was modified and used for extraction of both phenolic acids and flavonoids in *L. barbarum*. Initially, a 0.5-g powdered fruit sample of *L. barbarum* sample was mixed with 30 mL of 50% ethanol solution in a flask and shaken in a water bath at 90 °C for 2 h. After extraction, the extract was centrifuged at 4000 rpm for 30 min, the supernatant collected, evaporated to dryness and the residue dissolved in 5 mL of deionized water, followed by adding dilute hydrochloric acid to adjust the pH to 2.0 for purification using a SPE cartridge.

For purification, a Phenomenex Strata-X cartridge was preactivated with 4 mL of methanol and equilibrated with 4 mL of acidified deionized water (pH 2). Then 1 mL of acidified sample extract (pH 2) was poured into the cartridge, and the simple organic acids were removed by washing with 4 mL of acidified deionized water (pH 2), followed by eluting both phenolic acid and flavonoid fractions with 2 mL of methanol. Dilute hydrochloric acid was used for adjusting the pH of deionized water and sample extract. Methanol fraction collected was then evaporated to dryness under vacuum, dissolved in 1 mL of acetonitrile–water (1:1, v/v), filtered through a 0.2-μm membrane filter, and 20 μL injected for HPLC–DAD–ESI-MS analysis.

2.4. Separation and identification of phenolic acids and flavonoids

A binary solvent system of 0.5% (v/v) formic acid in deionized water (A) and acetonitrile–deionized water (94:6, v/v) (B) with the following gradient elution was developed: 98% A and 2% B in the beginning, maintained for 2 min, changed to 95% A in 25 min, 94% A in 30 min, 92% A in 40 min, 91.5% A in 48 min, 75% A in 70 min. The column temperature was at 30 °C, flow rate at 1.0 mL/min and detection using DAD at 280 nm. The peak purity of each peak was automatically determined by DAD. The retention factor (k) was calculated using the formula $k = (t_R - t_o)/t_o$, where t_R denotes retention time of sample components and t_o denotes retention time of sample solvent. The separation factor (α) was based on the formula $\alpha = k_2/k_1$, where k_1 and k_2 represents retention factor of two neighboring peaks.

The identification of phenolic acids and flavonoids was performed by comparing retention times and absorption spectra of unknown peaks with reference standards and those reported in the literature as well as co-chromatography with added standards. In addition, a triple quadrupole mass spectrometer with ESI (negative ion mode) was used for detection with the total ion scanning range being m/z 100–1200, nebulizer nitrogen gas flow rate 500 L/h, capillary voltage 2500 V, charging voltage 2500 V, cone voltage 40 V, ionization temperature 120 °C and desolvation temperature 450 °C.

For further identification of phenolic acids and flavonoid glycosides, the purified extract was hydrolyzed to obtain the free phenolic acids and flavonoid aglycones by modifying a method described by Lin et al. [21]. One milliliter of purified extract from SPE cartridge was mixed with 2 mL of 1.2N methanolic hydrochloric acid solution and the mixture was shaken in a water bath at 75 °C for 2 h for hydrolysis to occur. Then the hydrolyzate was evaporated to dryness under vacuum, redissolved in 1-mL of acetonitrile–water (1:1, v/v), and 20 μL was injected for HPLC–DAD–ESI-MS analysis.

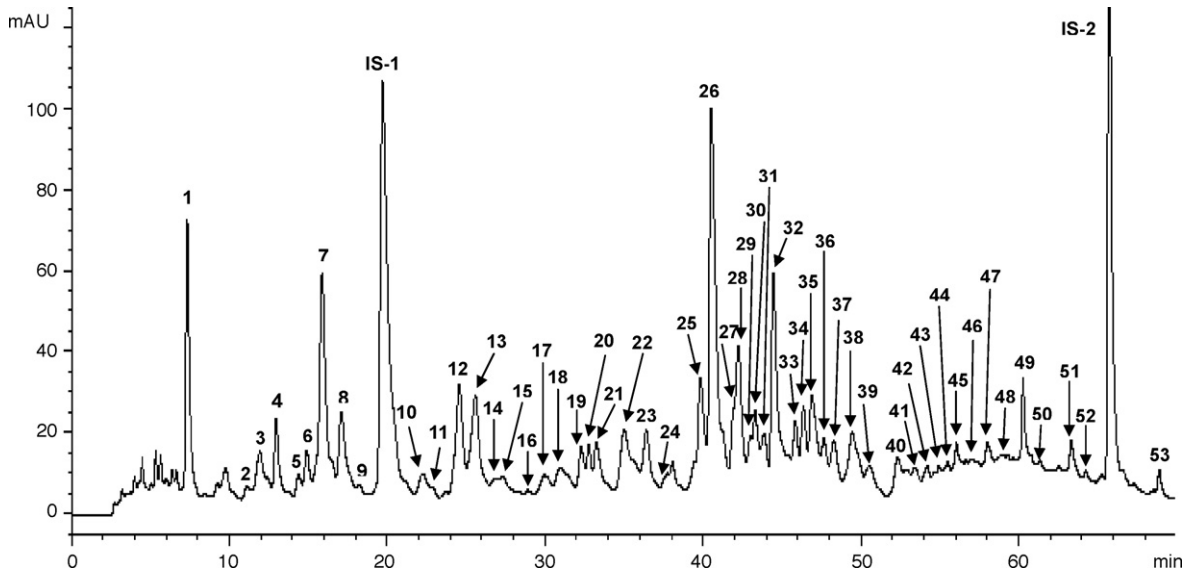

Fig. 1. HPLC chromatogram of simultaneous separation of phenolic acids and flavonoids extracted from *L. barbarum* fruits. IS-1, internal standard 3-hydroxybenzoic acid for phenolic acids; IS-2, internal standard hesperidin for flavonoids. The identification details of the peaks are shown in Table 1.

2.5. Quantitation of phenolic acids and flavonoids

Phenolic acids and flavonoids were quantified by using two different internal standards 3-hydroxybenzoic acid and hesperidin, respectively. A stock solution of 100 μg/mL was prepared by dissolving each phenolic acid and flavonoid standard in acetonitrile–water (1:1, v/v). Eight mass concentrations of 1, 2, 5, 8, 10, 12, 15 and 20 μg/mL each for caffeic acid, chlorogenic acid, p-coumaric acid, rutin and kaempferol-3-O-rutinoside were prepared separately, followed by mixing each phenolic acid solution with IS 3-hydroxybenzoic acid and flavonoid solution with IS hesperidin to provide a final IS mass concentration of 40 and 20 μg/mL, respectively, and 20 μL was injected into HPLC. The calibration curve of each phenolic acid and flavonoid standard was prepared by plotting mass concentration ratio (phenolic acid or flavonoid standard vs. IS) against its area ratio. The regression equations and correlation coefficient (R^2) for each standard curve were automatically determined using a Microsoft Excel XP software system. The regression equations for caffeic acid, chlorogenic acid, p-coumaric acid, rutin and kaempferol-3-O-rutinoside were $y = 6.0391x - 0.0232$, $y = 2.9678x - 0.0389$, $y = 9.1663x + 0.0028$, $y = 0.4488x - 0.0042$ and $y = 0.6812x + 0.0317$, respectively, with R^2 being all higher than 0.998. The mass fraction of phenolic acids and flavonoids in *L. barbarum* were quantified using the following formula:

$$\text{phenolic acid or flavonoid}\,(\mu g/g) = \frac{[(A/\text{RRF})/Ai] \times Ci \times \text{volume of extract/recovery}}{Ws}$$

where RRF, relative response factor = $(A/Ai)/(C/Ci)$; A, peak area of phenolic acid or flavonoid; Ai, peak area of IS; C, mass concentration of phenolic acid or flavonoid (μg/mL); Ci, mass concentration of IS (μg/mL); Ws, weight of sample (g). Because of unavailability of commercial standards, dicaffeoylquinic acid isomers were quantified based on the standard curve of chlorogenic acid, while quercetin glycosides and isorhamnetin-3-O-rutinoside were based on rutin. Since vanillic acid belong to hydroxybenzoic acid type of compounds, it was quantified by direct comparison of peak area of the IS 3-hydroxybenzoic acid.

For quantitation of phenolic acids in the hydrolysate of *L. barbarum*, external calibration curves were prepared for caffeic acid, chlorogenic acid, p-coumaric acid and ferulic acid without adding IS. Eight mass concentrations of 1, 2, 5, 8, 10, 12, 15 and 20 μg/mL of each phenolic acid were prepared separately and 20 μL was injected into HPLC. The regression equations obtained for caffeic acid, chlorogenic acid, p-coumaric acid and ferulic acid were $y = 49.779x - 18.882$, $y = 23.314x - 14.665$, $y = 75.104x - 31.111$ and $y = 42.278x - 14.613$, respectively, with R^2 being all higher than 0.996.

2.6. Quality control

Both detection limit (DL) and quantitation limit (QL) were determined based on a method described by the International Conference on Harmonization [22]. Four mass concentrations of 1, 2, 5 and 8 μg/mL for each phenolic acid and flavonoid standard were prepared, and 20 μL was injected into HPLC. Analysis was performed in triplicate and the calibration curves were obtained by plotting concentration against peak area. By calculating the mean of the slopes (S) and standard deviation of the intercepts (σ) from the three calibration curves, the DL and QL could be determined using the formula $3.3 \times (\sigma/S)$ and $10 \times (\sigma/S)$, respectively.

The recovery was determined by spiking two mass concentrations of 10 and 20 μg/mL for each standard (caffeic acid, chlorogenic acid, p-coumaric acid, rutin and kaempferol-3-O-rutinoside) to 0.5 g *L. barbarum* fruit sample for subsequent extraction and purification. After HPLC analysis, the recovery of each phenolic acid or flavonoid standard was determined based on the ratio of the standard concentration after HPLC (spiked amount minus original amount) and before HPLC (spiked amount). The intra-day variability was determined based on triplicate analyses each at morning, afternoon and evening for a total of 9 analyses within 1 day, whereas the inter-day variability was based on 3 analyses every week for a total of 9 analyses in 3 weeks.

2.7. Statistical analysis

Triplicate analyses were conducted for phenolic acids and flavonoids in *L. barbarum* samples and the mean values were subjected to analysis of variance and Duncan's multiple range test for mean comparison ($\alpha = 0.05$) by using Statistical Analysis System [23].

Table 1
Retention time (t_R), retention factor (k), separation factor (α), peak purity (%) and UV data of phenolic acids and flavonoids extracted from *L. barbarum* fruits.

Peak no.	Compound	Retention time (t_R, min)	Retention factor (k)[a]	Separation factor (α)[b]	Peak purity (%)	UV data (λ, nm)	
						Online	Reported
1	Arbutin	7.23	1.69	1.96 (1,2)[c]	94.8	284	283[d]
2	p-Coumaric acid derivative	11.61	3.32	1.96 (1,2)	95.6	230, 292, 306	291, 307[e]
3	p-Coumaric acid derivative	11.90	3.42	1.03 (2,3)	94.4	232, 292, 306	291, 307[e]
4	Hydroxycinnamic acid derivative	12.92	3.80	1.11 (3,4)	92.8	236, 286, 314	291, 319[e]
5	Dicaffeoylquinic acid	14.35	4.33	1.14 (4,5)	93.4	290, 318	298, 324[f]
6	p-Coumaric acid derivative	14.87	4.53	1.05 (5,6)	98.4	232, 292, 308	291, 307[e]
7	Unknown	15.83	4.88	1.08 (6,7)	92.8	294	–
8	Dicaffeoylquinic acid	17.07	5.35	1.10 (7,8)	92.6	296, 322	298, 324[f]
9	Vanillic acid	18.18	5.76	1.08 (8,9)	98.7	262, 294	263, 291[g]
IS1[h]	3-Hydroxybenzoic acid	19.67	6.31	1.10 (9,IS1)	98.6	–	–
10	Caffeic acid	22.19	7.25	1.15 (10,IS1)	95.4	238, 296, 322	291, 319[e]
11	p-Coumaric acid derivative	22.86	7.50	1.03 (10,11)	86.2	232, 292, 308	291, 307[e]
12	3-Caffeoylquinic acid (Chlorogenic acid)	24.53	8.12	1.08 (11,12)	95.3	232, 296, 320	294, 321[e]
13	Catechin or epicatechin derivative	25.53	8.49	1.05 (12,13)	92.6	230, 276	230, 278[e]
14	Hydroxycinnamic acid derivative	26.80	8.96	1.06 (13,14)	87.3	232, 296, 326	291, 319[e]
15	Quercetin-di-(rhamnohexoside)	27.31	9.16	1.02 (14,15)	93.1	262, 356	255, 355[i]
16	Quercetin-rhamno-di-hexoside	28.87	9.73	1.06 (15,16)	98.8	254, 264, 352	265, 350[i]
17	p-Coumaric acid derivative	29.92	10.12	1.04 (16,17)	96.6	232, 292, 308	291, 307[e]
18	Dicaffeoylquinic acid	30.91	10.49	1.04 (17,18)	95.2	296, 324	298, 324[f]
19	Catechin or epicatechin derivative	32.25	10.99	1.05 (18,19)	95.4	230, 278	230, 278[e]
20	Hydroxycinnamic acid derivative	32.72	11.16	1.02 (19,20)	98.0	238, 292, 318	291, 319[e]
21	Catechin or epicatechin derivative	33.20	11.34	1.02 (20,21)	95.3	230, 278	230, 278[e]
22	p-Coumaric acid	34.97	12.00	1.06 (21,22)	98.8	232, 290, 308	291, 307[e]
23	p-Coumaric acid derivative	36.36	12.52	1.04 (22,23)	96.4	232, 292, 308	291, 307[e]
24	Hydroxycinnamic acid derivative	37.69	13.01	1.04 (23,24)	86.7	232, 294, 318	291, 319[e]
25	Catechin or epicatechin derivative	39.80	13.80	1.06 (24,25)	97.0	230, 278	230, 278[e]
26	Hydroxycinnamic acid derivative	40.47	14.04	1.02 (25,26)	95.8	234, 286, 316	291, 319[e]
27	Quercetin-rhamno-di-hexoside	41.87	14.57	1.04 (26,27)	90.7	256, 266, 354	265, 350[i]
28	Hydroxycinnamic acid derivative	42.20	14.69	1.01 (27,28)	94.6	232, 284, 316	291, 319[e]
29	Hydroxycinnamic acid derivative	42.96	14.97	1.02 (28,29)	88.2	232, 290, 316	291, 319[e]
30	Hydroxycinnamic acid derivative	43.25	15.08	1.01 (29,30)	98.2	234, 286, 316	291, 319[e]
31	Hydroxycinnamic acid derivative	43.81	15.29	1.01 (30,31)	85.4	232, 286, 318	291, 319[e]
32	p-Coumaric acid derivative	44.43	15.52	1.02 (31,32)	95.4	234, 290, 310	291, 307[e]
33	p-Coumaric acid derivative	45.81	16.03	1.03 (32,33)	98.2	232, 290, 310	291, 307[e]
34	p-Coumaric acid derivative	46.32	16.22	1.01 (33,34)	99.2	234, 290, 310	291, 307[f]
35	Hydroxycinnamic acid derivative	46.85	16.42	1.01 (34,35)	93.6	234, 296, 322	291, 319[f]
36	p-Coumaric acid derivative	47.62	16.70	1.02 (35,36)	97.4	234, 292, 310	291, 307[e]
37	p-Coumaric acid derivative	48.25	16.94	1.01 (36,37)	98.6	236, 290, 310	291, 307[e]
38	Hydroxycinnamic acid derivative	49.41	17.34	1.02 (37,38)	92.4	234, 288, 318	291, 319[e]
39	Hydroxycinnamic acid derivative	50.48	17.77	1.02 (38,39)	96.1	236, 294, 316	291, 319[e]
40	Hydroxycinnamic acid derivative	52.29	18.44	1.04 (39,40)	84.4	232, 292, 318	291, 319[e]
41	Hydroxycinnamic acid derivative	53.39	18.85	1.02 (40,41)	90.8	234, 292, 316	291, 319[e]
42	p-Coumaric acid derivative	54.17	19.14	1.02 (41,42)	95.6	242, 294, 310	291, 307[e]
43	p-Coumaric acid derivative	54.90	19.41	1.01 (42,43)	99.5	232, 290, 308	291, 307[e]
44	Hydroxycinnamic acid derivative	55.45	19.61	1.01 (43,44)	99.8	238, 296, 316	291, 319[e]
45	Hydroxycinnamic acid derivative	56.00	19.82	1.01 (44,45)	97.8	236, 294, 316	291, 319[e]
46	Quercetin-di-(rhamno)-hexoside	56.88	20.14	1.02 (45,46)	92.0	256, 266, 354	256, 354[j]
47	Hydroxycinnamic acid derivative	57.95	20.54	1.02 (46,47)	98.1	240, 292, 318	291, 319[e]
48	Quercetin-rhamno-di-hexoside	59.26	21.03	1.02 (47,48)	93.7	256, 266, 354	265, 350[i]
49	Quercetin-3-O-rutinoside (Rutin)	60.23	21.39	1.02 (48,49)	94.5	256, 265, 354	256, 354[j]
50	Hydroxycinnamic acid derivative	61.29	21.78	1.02 (49,50)	98.8	234, 294, 318	291, 319[e]
51	Kaempferol-3-O-rutinoside	63.29	22.53	1.03 (50,51)	90.6	266, 348	264, 348[j]
52	Isorhamnetin-3-O-rutinoside	64.19	22.86	1.01 (51,52)	99.4	256, 266, 352	254, 355[g]
IS2[k]	Hesperidin	65.70	23.42	1.02 (52,IS2)	94.8	–	–
53	Hydroxycinnamic acid derivative	68.86	24.60	1.05 (53,IS2)	97.7	234, 296, 318	291, 319[e]

[a] Retention factor $(k) = (t_R - t_o)/t_o$, where t_R denotes retention time of sample components and t_o denotes retention time of sample solvent.
[b] Separation factor $(\alpha) = k_2/k_1$, where k_1 and k_2 represent retention factor of neighboring peaks.
[c] Numbers in parentheses represent peak numbers.
[d] Tentatively identified based on the UV data for arbutin cited by Abad-García et al. [25].
[e] Tentatively identified based on the UV data for caffeic acid, p-coumaric acid and chlorogenic acid cited by Sakakibara et al. [8].
[f] Tentatively identified based on the UV data for dicaffeoylquinic acid cited by Truong et al. [26].
[g] Tentatively identified based on the UV data for vanillic acid and isorhamnetin-3-O-rutinoside cited by Monagas et al. [27].
[h] IS1: internal standard for phenolic acids.
[i] Tentatively identified based on the UV data for quercetin-rhamno-di-hexoside and quercetin-di-(rhamnohexoside) cited by Tiberti et al. [28].
[j] Tentatively identified based on the UV data for quercetin-di-(rhamno)-hexoside, quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside cited by Kao et al. [24].
[k] IS2: internal standard for flavonoids.

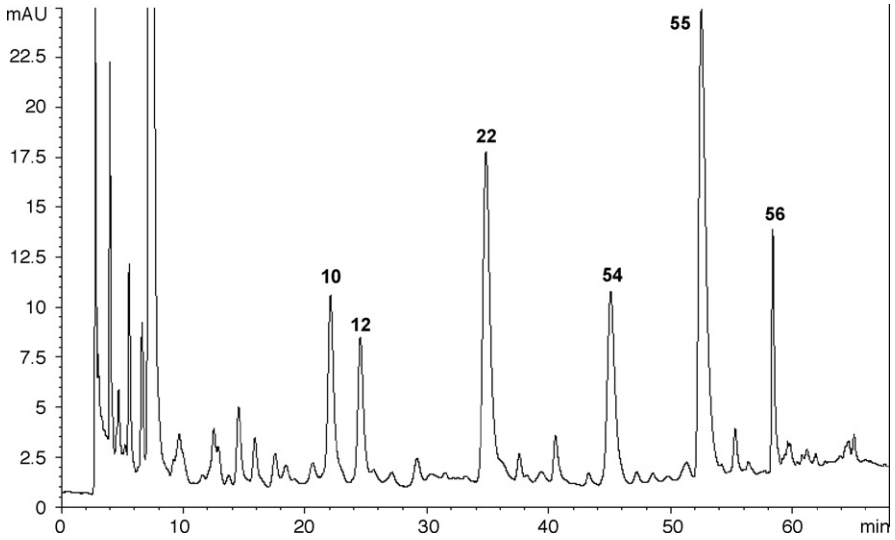

Fig. 2. HPLC chromatogram of the hydrolyzed extract of *L. barbarum* fruits developed under the same gradient solvent system as that of original extract (shown in Fig. 1). The identification details of peaks are shown in Table 2.

3. Results and discussion

3.1. HPLC analysis of phenolic acids and flavonoids

Initially, several binary and ternary solvent systems in isocratic or gradient mode were compared with respect to separation efficiency of phenolic acids and flavonoids. In addition, several modifiers such as formic acid, acetic acid, phosphoric acid, citric acid and trifluoroacetic acid were added to the mobile phase to enhance peak resolution, as it has been well established that the incorporation of acidic compounds may reduce peak tailing during separation of flavonoids [15]. Kao et al. [24] reported that the addition of tetrahydrofuran (THF) could improve the selectivity of mobile phase for aromatic compounds. However, we found that the incorporation of THF failed to adequately resolve both phenolic acids and flavonoids extracted from *L. barbarum* fruits, which may be attributed to the inability of THF to donate a proton for ionization of target compounds. After several trial studies, a binary solvent system with formic acid or phosphoric acid as modifier gave better peak resolution, but the former was chosen as phosphoric acid may contaminate the ion source of LC–MS more readily [24]. A gradient solvent system as described in Section 2.4 was developed and a total of 52 phenolic acids and flavonoids were resolved within 70 min (Fig. 1). Of the 52 compounds separated, 15 were positively identified by both UV and mass data, whereas the remaining 37 were tentatively identified based on comparison of their UV data with those reported in the literature. Table 1 shows the retention time (t_R), retention factor (k), separation factor (α) and peak purities for the phenolic acids and flavonoids extracted from *L. barbarum*, which ranged from 7.23 to 68.86 min, 1.69 to 24.60, 1.01 to 1.96 and 84.4 to 99.8%, respectively. The α values were higher than 1 for all the peaks, revealing a good selectivity of mobile phase to both phenolic acids and flavonoids was attained. With the exception of peaks 11, 14, 24, 29, 31 and 40, the purities of all the other peaks were higher than 90%.

The tentative identification of all the 52 peaks based on UV data along with their corresponding reported values for comparison [8,24–28] was summarized in Table 1. Several peaks showed similar absorption maximum values that are characteristic of hydroxycinnamic acid, p-coumaric acid or catechin/epicatechin. Peaks 4, 14, 20, 24, 26, 28–31, 35, 38–41, 44, 45, 47, 50 and 53 were identified to be derivatives of hydroxycinnamic acids as their online absorption maxima (314–326 nm) were nearly identical to that of hydroxycinnamic acids such as caffeic acid or ferulic acid [8]. Likewise, peaks 2, 3, 6, 11, 17, 23, 32–34, 36, 37, 42 and 43 were detected to be p-coumaric acid derivatives as their absorption maxima ranged between 306 and 310 nm, which is typical for p-coumaric acid [8]. Though p-coumaric acid belongs to the class of hydroxycinnamic acids, its derivatives are grouped separately as their absorption spectra could be clearly distinguished from the other hydroxycinnamic acids derivatives. Peaks 13, 19, 21 and 25 all showed absorption spectral values at 230 and 278 nm, which matched with

Table 2
Identification data and content (μg/g) of phenolic acids in the hydrolyzed extract of *L. barbarum* fruits.

Peak no.	Compound	Retention time (t_R, min)	UV data (λ, nm)		[M−H]⁻		Content (μg/g)[a]
			Online	Reported	Online	Reported	
10	Caffeic acid[b]	22.03	244, 298, 322	241, 291, 319[c]	178.9	179.3[d]	106.1 ± 0.7
12	Chlorogenic acid[b]	24.50	242, 298, 324	241, 294, 321[c]	353.1	353.3[d]	197.0 ± 1.0
22	p-Coumaric acid[b]	34.82	232, 290, 310	226, 291, 307[c]	163.1	163.1[e]	151.4 ± 1.4
54	Ferulic acid[b]	45.03	240, 298, 326	239, 291, 325[c]	193.3	192.9[f]	132.0 ± 0.9
55	Hydroxycinnamic acid derivatives	52.47	232, 290, 320	241, 291, 319[c]	–	–	–
56	Hydroxycinnamic acid derivatives	58.35	238, 296, 318	241, 291, 319[c]	–	–	–

[a] Compounds conclusively identified by comparison with authentic standards.
[b] Mean of triplicate measurements ± standard deviation.
[c] Tentatively identified on comparison with UV and [M−H]⁻ data reported by Sakakibara et al. [8].
[d] Tentatively identified on comparison with UV and [M−H]⁻ data reported by Truong et al. [26].
[e] Tentatively identified on comparison with UV and [M−H]⁻ data reported by Monagas et al. [27].
[f] Tentatively identified on comparison with UV and [M−H]⁻ data reported by Ayaz et al. [30].

Table 3
Mass spectral data and content (μg/g) of positively identified phenolic acids and flavonoid glycosides in *L. barbarum* fruits.

Peak no.	Compound	Mass data (Online)		Mass data (Reported)		Contents[a]
		$[M-H]^-$	Fragments	$[M-H]^-$	fragments	
5	Dicaffeoylquinic acid	515	353 [M−H-Caffeoyl], 191 [M−H-2Caffeoyl], 179 [M−H-Caffeoyl-Quinic]	515[b]	353, 191, 179[b]	28.2 ± 0.3
8	Dicaffeoylquinic acid	515	353, 191, 179	515[b]	353, 191, 179[b]	134.8 ± 1.1
9	Vanillic acid[c]	167	–	167[d]	–	22.8 ± 0.4
10	Caffeic acid[c]	179	–	179[d]	–	23.7 ± 0.1
12	3-Caffeoylquinic acid (Chlorogenic acid)[c]	353	191 [M−H-Caffeoyl], 179 [M−H-Quinic]	353[d]	191, 179[d]	237.0 ± 1.5
15	Quercetin-di-(rhamnohexoside)	917	609 [M−H-Rhamnose-Hexose], 301 [M−H-2Rhamnose-2Hexose]	917[e]	301, 271[e]	117.5 ± 0.6
16	Quercetin-rhamno-di-hexoside	771	301 [M−H-Rhamnose-2Hexose]	771[e]	300, 271[e]	56.5 ± 0.4
18	Dicaffeoylquinic acid	515	353, 191, 179	515[b]	353, 191, 179[b]	87.1 ± 1.2
22	p-coumaric acid[c]	163	–	163[d]	–	64.0 ± 0.4
27	Quercetin-rhamno-di-hexoside	771	301	771[e]	300, 271[e]	310.1 ± 2.2
46	Quercetin-di-(rhamno)-hexoside	755	609 [M−H-Rhamnose], 301 [M−H-Rhamnose-Hexose]	755[f]	609[f]	116.8 ± 1.0
48	Quercetin-rhamno-di-hexoside	771	301	771[e]	300, 271[e]	72.0 ± 0.6
49	Quercetin-3-O-rutinoside (Rutin)[c]	609	463 [M−H-Rhamnose], 301 [M−H-Rhamnose-Glucose]	609[f]	463, 301[f]	281.3 ± 2.1
51	Kaempferol-3-O-rutinoside[c]	593	447 [M−H-Rhamnose], 284 [M-2H-Rhamnose-Glucose]	593[f]	447, 284[f]	97.7 ± 0.9
52	Isorhamnetin-3-O-rutinoside	623	477 [M−H-Rhamnose], 315 [M−H-Rhamnose-Glucose]	623[e]	315[e]	72.1 ± 1.4

[a] Mean of triplicate measurements ± standard deviation.
[b] Identification confirmed by comparison with mass data reported by Bravo et al. [29].
[c] Compounds conclusively identified by comparison with authentic standards.
[d] Identification confirmed by comparison with mass data reported by Monagas et al. [27].
[e] Identification confirmed by comparison with mass data reported by Tiberti et al. [28].
[f] Identification confirmed by comparison with mass data reported by Kao et al. [24].

that of catechin or epicatechin [8], indicating the possible presence of catechin or epicatechin derivatives. These peak assignments were further substantiated by identifying free phenolic acids in the hydrolysate and comparing with that in the original extract (Figs. 1 and 2). On hydrolysis of *L. barbarum* extract, numerous peaks in Fig. 1 were simplified to only 6 major peaks in Fig. 2, of which 4 peaks were identified to be caffeic acid (peak 10), chlorogenic acid (peak 12), p-coumaric acid (peak 22) and ferulic acid (peak 54) by comparing their retention times, absorption spectra and $[M-H]^-$ values with those of authentic standards [8,26,27,30], while peaks 55 and 56 were identified to be hydroxycinnamic acid derivatives based on their absorption spectra (Table 2). The contents of caffeic acid, p-coumaric acid and ferulic acid in the hydrolysate were quantified by using the external calibration curves prepared with the respective standards. Compared to the original extract (23.7 and 64.0 μg/g), the mass fractions of caffeic acid and p-coumaric were higher in the hydrolysate (106.1 and 151.4 μg/g), revealing the presence of several glycoside conjugates of caffeic acid and p-coumaric acid. It is interesting to note that ferulic acid, which was not present in the extract, was identified in the hydrolysate (132.0 μg/g), implying the presence of ferulic acid as glycoside conjugates. Thus, it may be postulated that several tentatively identified hydroxycinnamic acid and p-coumaric acid derivatives may belong to phenolic acid glycosides and were present as the conjugates of caffeic acid, p-coumaric acid, ferulic acid and other hydroxycinnamic acid derivatives.

Table 3 shows the mass spectral data including $[M-H]^-$ value and fragment ions for positive identification of 7 phenolic acids and 8 flavonoid glycosides along with the corresponding data reported in the literature for comparison [24,27–29]. Peaks 5, 8, 12 and 18 were identified as phenolic acid esters formed by a combination of caffeic acid and quinic acid, of which peaks 5, 8 and 18 all showing a $[M-H]^-$ value at m/z 515 was identified as dicaffeoylquinic acid isomers based on the fragment ions at m/z 353, 191 and 171 obtained due to loss of 1 caffeoyl, 2 caffeoyl and 1 caffeoyl plus 1 quinic acid moieties, respectively, whereas 3-caffeoylquinic acid (chlorogenic acid) was assigned for peak 12 as a $[M-H]^-$ value at m/z 353 and fragments at m/z 191 and 179 were obtained [27,29]. Peaks 9, 10 and 22 were determined to be vanillic acid, caffeic acid and p-coumaric acid based on their unique absorption maximum at 262, 322 and 308 nm (Table 1) as well as $[M-H]^-$ values at m/z 167, 179 and 163 (Table 3), respectively [8,27]. Additionally, 8 flavonoid glycosides were identified, with peaks 16, 27 and 48 all showing a $[M-H]^-$ value at m/z 771 were determined to be quercetin-rhamno-di-hexoside based on the fragment at m/z 301 obtained due to loss of 1 rhamnose and 2 hexose. Likewise, peaks 15 and 46 with a $[M-H]^-$ of m/z 917 and 755, respectively, yielded the same fragments at m/z 609 and 301 and were identified as quercetin-di-(rhamnohexoside) and quercetin-di-(rhamno)-hexoside (Table 3) [28]. Peaks 49, 51 and 52 with a $[M-H]^-$ value at m/z 609, 593 and 623 were identified as quercetin-3-O-rutinoside (rutin), kaempferol-3-O-rutinoside and isorhamnetin-3-O-rutinoside, respectively, based on the fragments ions obtained due to loss of 1 rhamnose (m/z 463, 447 and 477) and 1 rhamnose plus 1 glucose (m/z 301, 284 and 315) (Table 3) [24,27]. For further identification of flavonoid glycosides, *L. barbarum* extract was hydrolyzed with 1.2N methanolic hydrochloric acid for 2 h to yield three aglycones, which were identified to be quercetin, kaempferol and isorhamnetin by comparing their retention times, absorption and mass spectra with that of authentic standards. The absorption spectra and mass data of all the phenolic acids and flavonoid glycosides correlated well with the typical values reported in the literature (Tables 1 and 3) [8,24–29]. In addition, the identification of vanillic acid (peak 9), caffeic acid (peak 10), chlorogenic acid (peak 12), p-coumaric acid (peak 22), rutin (peak 49) and kaempferol-3-O-rutinoside (peak 51) were further

confirmed by comparing their retention times, absorption spectra and mass data with those of authentic standards as well as co-chromatography with added standards. Among the 15 positively identified compounds, quercetin-rhamno-di-hexoside was present in largest mass fraction (438.6 μg/g), followed by quercetin-3-O-rutinoside (281.3 μg/g), dicaffeoylquinic acid isomers (250.1 μg/g), chlorogenic acid (237.0 μg/g), quercetin-di-(rhamnohexoside) (117.5 μg/g), quercetin-di-(rhamno)-hexoside (116.8 μg/g), kaempferol-3-O-rutinoside (97.7 μg/g), isorhamnetin-3-O-rutinoside (72.1 μg/g), p-coumaric acid (64.0 μg/g), caffeic acid (23.7 μg/g) and vanillic acid (22.8 μg/g) (Table 3). The remaining 37 compounds were not quantified as their identity requires further investigation.

Epidemiological studies have consistently shown a positive correlation between intake of fruits and vegetables and risk reduction of heart disease, cancers and degenerative diseases [31–33]. This beneficial effect can be attributed to the presence of various phytochemicals such as phenolic acids and flavonoids in plants. In this study, we proved *L. barbarum* fruits to be rich in hydroxycinnamic acids and their derivatives, which would exert a high antioxidative activity amid the presence of CH=CH–COOH group [31]. In addition, Islam et al. [32] pointed out that the ortho-dihydroxy group in the catechol ring of caffeic acid is responsible for the antioxidant activity. Besides caffeic acid, the other hydroxycinnamic acids like ferulic acid and p-coumaric acid and their conjugates were demonstrated to possess high antioxidant activity [31,33]. On the other hand, a significant mass fraction of flavonoid glycosides (mainly quercetin glycosides) found in *L. barbarum* fruits should contribute to the overall biological activity as well [4–6].

3.2. Quality control

According to the methods described in the preceding section, the detection and quantitation limits of caffeic acid, chlorogenic acid, p-coumaric acid, rutin and kaempferol-3-O-rutinoside were determined to be 0.03 and 0.09 μg/mL, 0.05 and 0.15 μg/mL, 0.03 and 0.09 μg/mL, 0.27 and 0.81 μg/mL, and 0.23 and 0.69 μg/mL, respectively, while the coefficient of variation (%) for the intra- and inter-day variability were 1.21 and 2.56, 0.62 and 1.98, 0.84 and 2.13, 1.44 and 2.83, and 1.10 and 2.47. Based on two standard mass concentrations (10 and 20 μg/mL) and three determinations of each, the average recovery of caffeic acid, chlorogenic acid, p-coumaric acid, rutin and kaempferol-3-O-rutinoside were determined to be 94.4, 92.4, 95.3, 90.0 and 91.5%, respectively. A less than 100% recovery may be probably due to a high temperature treatment at 90 °C to accelerate extraction of phenolic compounds within a short period of time.

All in all, an HPLC–DAD–ESI-MS method was developed to simultaneously separate 52 phenolic acids and flavonoids within 70 min, of which 15 were positively identified based on MS/MS data. In comparison, the total number of phenolic compounds separated and identified in our study was higher than that reported by some previous studies on *Lycium* fruits [7,34]. By employing a binary gradient solvent system of acetonitrile/water/acetic acid at varying proportions, Qian et al. [7] could separate only 7 phenolic acids and flavonoids from *L. chinense* Mill, which included protocatechuic acid, chlorogenic acid, rutin, hyperoside, hesperidin, morin and quercetin. Likewise, Le et al. [34] identified only 3 flavonoids, namely, myricetin, quercetin and kaempferol from deglycosylated extract of *L. barbarum* fruits by using a C18 column and a gradient mobile phase of acetonitrile and 1% acetic acid. In both studies, the phenolic composition was different from ours, which may be accounted for by the variation in *Lycium* species as well as difference in extraction and purification conditions Additionally, our method was superior in terms of separation number and total run time when compared to other reports. Chen et al. [35] separated a total of 9 phenolic acid and 4 flavonoid standards in two fractions, both of which injected separately into HPLC and a total run time of 72 min was observed, but a poor resolution occurred when applied to cranberry juice. Likewise, by injecting two fractions separately into HPLC with varying gradient mobile phase conditions, a total of 145 min was required for separation of 43 phenolic acids and flavonoids from dandelion root and herb [17]. In another study, Wang and Huang [15] could separate 8 flavonoids and 1 phenolic acid in wine within 17 min, however, the separation number is inadequate. For separation of phenolic compounds from Greek aromatic plants, Proestos et al. [36] employed a ternary solvent system of 1% acetic acid, 6% acetic acid and water–acetonitrile (65:30, v/v) and resolved a total of 16 phenolic acids and flavonoids. Yet, the total run time was 105 min, and no data on recovery as well as limits of detection and quantitation were provided. In a recent study by Askun et al. [37], 3 phenolic acids and 13 flavonoids were separated from two thyme species within 80 min, however, the method was not validated with recovery and reproducibility data.

4. Conclusion

Both phenolic acids and flavonoids were isolated from the fruits of *L. barbarum* by extraction using 50% ethanol and purification by a Strata-X polymeric reversed phase SPE cartridge. A total of 52 phenolic acids and flavonoids were simultaneously separated by using a Vydac C18 column and a gradient mobile phase of 0.5% (v/v) formic acid in deionized water and acetonitrile-deionized water (94:6, v/v) within 70 min, of which 15 compounds including 7 phenolic acids and 8 flavonoids glycosides were positively detected by both absorption spectra and mass data, while the remaining 37 were tentatively identified based on comparison of their absorption maxima with reported values, as well as characteristic absorption spectra and mass data of the hydrolysate of *L. barbarum* extract analyzed under the same gradient solvent system. The method developed in this study may be applied to determine phenolic acid and flavonoid composition in both foods and nutraceuticals.

References

[1] T. Tanaka, T. Kojima, T. Kawamori, A. Wang, M. Suzui, K. Okamoto, H. Mori, Carcinogenesis 14 (1993) 1321–1325.
[2] B.S. Han, C.B. Park, N. Takasuka, A. Naito, K. Sekine, E. Nomura, H. Taniguchi, T. Tsuno, H.A. Tsuda, Cancer Sci. 92 (2001) 404–409.
[3] R. Tsao, Z. Deng, J. Chromatogr. B 812 (2004) 85–99.
[4] E.E. Deschner, J. Ruperto, G. Wong, H.L. Newmark, Carcinogenesis 12 (1991) 1193–1196.
[5] D.R. Ferry, A. Smith, J. Malkhandi, D.W. Fyfe, P.G. de Takats, D. Anderson, Clin. Cancer Res. 2 (1996) 659–668.
[6] M.A. Pereira, C.J. Grubbs, L.H. Barnes, H. Li, G.R. Olson, I. Eto, M. Juliana, L.M. Whitaker, G.J. Kelloff, V.E. Steele, R.A. Lubet, Carcinogenesis 17 (1996) 1305–1311.
[7] J.Y. Qian, D. Liu, A.G. Huang, Food Chem. 87 (2004) 283–288.
[8] H. Sakakibara, Y. Honda, S. Nakagawa, H. Ashida, K. Kanazawa, J. Agric. Food Chem. 51 (2003) 571–581.
[9] V. Bankova, R. Christov, G. Stoev, S. Popov, J. Chromatogr. 607 (1992) 150–153.
[10] I. Molnár-Perl, Z. Füzfai, J. Chromatogr. A 1073 (2005) 201–227.
[11] T.M. Upson, R.J. Grayer, J.R. Greenham, C.A. Williams, F. Al-Ghamdi, F.H. Chen, Biochem. System Ecol. 28 (2000) 991–1007.
[12] J. Onyilagha, A. Bala, R. Hallett, M. Gruber, J. Soroka, N. Westcott, Biochem. System Ecol. 31 (2003) 1309–1322.
[13] J.F. Stevens, H. Hart, E.T. Elema, A. Bolck, Phytochemistry 41 (1996) 503–512.
[14] G.J. Soleas, J. Yan, D.M. Goldberg, J. Chromatogr. B 757 (2001) 161–172.
[15] S.P. Wang, K.J. Huang, J. Chromatogr. A 1032 (2004) 273–279.
[16] C.T. da Costa, D. Horton, S.A. Margolis, J. Chromatogr. A 881 (2000) 403–410.
[17] K. Schütz, D.R. Kammerer, R. Carle, A. Schieber, Rapid Commun. Mass Spectrom. 19 (2005) 179–186.
[18] L. Gan, S.H. Zhang, X.L. Yang, H.B. Xuc, Int. Immunopharmacol. 4 (2004) 563–569.
[19] K.T. Ha, S.J. Yoon, D.Y. Choi, D.W. Kim, J.K. Kim, C.H. Kim, J. Ethnopharmacol. 96 (2005) 529–535.
[20] B.S. Inbaraj, H. Lu, C.F. Hung, W.B. Wu, C.L. Lin, B.H. Chen, J. Pharm. Biomed. Anal. 47 (2008) 812–818.
[21] L.Z. Lin, S. Mukhopadhyay, R.J. Robbins, J.M. Harnly, J. Food Compos. Anal. 20 (2007) 361–369.

[22] International Conference on Harmonization (ICH). Guideline on the validation of analysis procedures: methodology Q2B, 1996.
[23] Statistical Analysis Software (SAS), SAS Procedures and SAS/Graph User's Guide, Version 6, SAS Institute, Inc., Cary, NC, 2004.
[24] T.H. Kao, S.C. Huang, B.S. Inbaraj, B.H. Chen, Anal. Chim. Acta 626 (2008) 200–211.
[25] B. Abad-García, L.A. Berrueta, D.M. López-Márquez, I. Crespo-Ferrer, B. Gallo, F. Vicente, J. Chromatogr. A 1154 (2007) 87–96.
[26] V.D. Truong, R.F. McFeeters, R.T. Thompson, L.L. Dean, B. Shofran, J. Food Sci. 72 (2007) C343–C349.
[27] M. Monagas, I. Garrido, R. Lebrón-Aquilar, B. Bartolome, C. Gómex-Cordovés, J. Agric. Food Chem. 55 (2007) 8498–8507.
[28] L.A. Tiberti, J.H. Yariwake, K. Ndjoko, K. Hostettmann, J. Chromatogr. B 846 (2007) 378–384.
[29] L. Bravo, L. Goya, E. Lecumberri, Food Res. Int. 40 (2007) 393–405.
[30] F.A. Ayaz, S. Hayirliaglu-Ayaz, J. Gruz, O. Novak, M. Strnad, J. Agric. Food Chem. 53 (2005) 8116–8122.
[31] K.H. Kim, R. Tsao, R. Yang, S.W. Cui, Food Chem. 95 (2006) 466–473.
[32] M.S. Islam, M. Yoshimoto, O. Yamakawa, J. Food Sci. 68 (2003) 111–116.
[33] P. Mattila, J. Hellström, R. Törrönen, J. Agric. Food Chem. 54 (2006) 7193–7199.
[34] K. Le, F. Chiu, K. Ng, Food Chem. 105 (2007) 353–363.
[35] H. Chen, Y. Zuo, Y. Deng, J. Chromatogr. A 913 (2001) 387–395.
[36] C. Proestos, I.S. Boziaris, G.J.E. Nychas, M. Komaitis, Food Chem. 95 (2006) 664–671.
[37] T. Askun, G. Tumen, F. Satil, M. Ates, Food Chem. 116 (2009) 289–294.