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Liquid chromatographic–photodiode array mass spectrometric analysis of dietary phytoestrogens from human urine and blood

Adrian A. Franke*, Laurie J. Custer, Lynne R. Wilkens, Loïc Le Marchand, Abraham M.Y. Nomura, Marc T. Goodman, Laurence N. Kolonel

Cancer Research Center of Hawai'i, 1236 Lauhala Street, Honolulu, HI 96813, USA

Abstract

Dietary phytoestrogens have been implicated in the prevention of chronic diseases. However, it is uncertain whether the phytoestrogens or the foods associated with phytoestrogens account for the observed effects. We report here a new liquid chromatography photodiode array mass spectrometry (LC–PDA-MS) assay for the determination of nanomolar amounts of the most prominent dietary phytoestrogens (genistein, dihydrogenistein, daidzein, dihydrodaidzein, glycitein, *O*-desmethylangolensin, hesperetin, naringenin, quercetin, enterodiol, enterolactone) in human plasma or serum and urine. This assay was found to be suitable for the assessment of quercetin exposure in an onion intervention study by measuring urinary quercetin levels. Other successful applications of this assay in clinical and epidemiologic studies validated the developed method and confirmed previous results on the negative association between urinary isoflavone excretion and breast cancer risk.

Keywords: Phytoestrogens

1. Introduction

A plant-based diet is suspected to prevent chronic diseases. However, which plant constituent explains this effect remains unknown [1,2]. Phytoestrogens, a group of plant molecules with estrogenic or antiestrogenic effects [3–5] that includes isoflavonoids, flavonoids, lignans and other phenolic agents [6,7], occur in many plant-based foods and are implicated in the prevention of chronic diseases, particularly cancer [8–11]. Increasing evidence suggests that consumption of soy products, the major source of dietary isoflavonoid exposure [9,12,13], might protect against heart disease, osteoporosis and cancer [14–16]. Significant effects of soy intake have been reported against breast, prostate and colon cancer [17,18], with potential effects also against endometrial and bladder cancer [19,20]. The risk of developing these chronic conditions is generally higher in Western populations than in Asian countries where consumption of soy is high [9,21,22]. Protection from risk in these Asian populations, however, decreases after migration to the West, suggesting a predominant influence of environmental factors [23,24]. Similarly, a flavonoid-rich diet might protect against heart disease [25–27], and against lung [28–30], stomach [31] and breast cancer [32,33]. However, these findings are inconsistent [27] and highly debated [34,35]. Whether the phytoestrogens or the complex combination of other phytochemical and

*Corresponding author. Tel.: +1-808-586-3008; fax: +1-808-586-2970.

E-mail address: adrian@crch.hawaii.edu (A.A. Franke).

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environmental factors associated with phytoestrogen-rich foods, or both, account for the observed effects remains to be determined. Liquid chromatography–mass spectrometry (LC–MS) combines the advantages of LC, i.e. high speed separation and low to no sample preparation requirements, with those of MS, i.e. high selectivity and sensitivity with exceptional diagnostic ability. The favored interface from the liquid domain of the chromatographic system to the gas domain of the mass spectrometer is electrospray ionization (ESI) due to the lack of thermal stress to the analytes. The only requirement of this technique is partial ionization of the analyte in solution. These convincing benefits led to considerable acceptance of LC–MS in analytical chemistry, including the analysis of isoflavonoids, which were determined most recently by this technique from foods [36–38], human urine and plasma [39–41], and rat blood and tissue [42,43]. In previously reported LC–MS assays of phytoestrogens in human body fluids, either flavonoids [44] or isoflavonoids and lignans [41] were excluded. Extending our previous LC methods for isoflavonoid quantitation based on photo-diode array (PDA) detection [45], we report here on newly designed LC–PDA–ESI–MS techniques for the determination of nanomolar amounts of the most prominent dietary phytoestrogens, such as isoflavonoids (daidzein, genistein, glycitein and their metabolites dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolensin), flavonoids (hesperetin, naringenin, quercetin) and mammalian lignans (enterodiol, enterolactone), in human plasma or serum and urine in order to assess the yet uncertain role of dietary phytoestrogens in protection against cancer and other diseases.

2. Experimental

2.1. Apparatus

Liquid chromatography photo-diode array electrospray mass spectrometry (LC–PDA–ESI–MS) was carried out with a Spectra-Physics designed quaternary solvent delivery liquid chromatography system with multiple channel diode-array detection and a quadrupole ion trap mass spectrometer model ‘‘LCQ Classic’’ equipped with an electrospray ionization unit (Thermo Finnigan, San Jose, CA, USA). Absorbance readings were obtained from a DU-62 spectrophotometer (Beckman, Fullerton, CA, USA).

2.2. Chemicals

Methanol, acetic acid, dimethyl sulfoxide (DMSO), diethylether and all other solvents were analytical grade or HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Daidzein, glycitein, genistein, dihydrogenistein, dihydrodaidzein, *O*-desmethylangolensin, enterodiol, and enterolactone were purchased from Dr. K. Wähälä, University of Helsinki, Finland. Hesperetin, naringenin, taxifolin, and quercetin were purchased from Sigma (St. Louis, MO, USA), formononetin was obtained from Aldrich (Milwaukee, WI, USA), and β-glucuronidase isolated from *Escherichia coli* (200 U/mL at 25 °C) and arylsulfatase isolated from *Helix pomatia* (5 U/mL at 25 °C) were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Hydrobond PS HPLC columns were obtained from MAC-MOD Analytical (Chadds Ford, PA, USA).

2.3. Extraction

Extraction of urines was performed as described previously [45–48]. In brief, frozen urine was thawed, stirred and centrifuged, followed by mixing 0.25 mL clear supernatant with 50 μL 0.5 *M* triethylamine acetate (pH 7.0), 5 μL β-glucuronidase and 5 μL arylsulfatase and incubating for 1 h at 37 °C. This mixture was extracted three times with 2.0 mL ethyl ether after addition of 50 μL formononetin (10 ppm dissolved in methanol) as internal standard. Taxifolin was used as internal standard if quercetin was included in the assay. After centrifugation the organic phases were combined and dried under nitrogen. The dry extract was redissolved in 0.25 mL methanol by vortexing followed by mixing with 0.25 mL 0.2 *M* acetate buffer (pH 5). The resulting clear solution was analyzed immediately by injecting 20 μL into the LC system or stored at −20 °C until analyzed.

Extraction from serum or plasma was performed with slight modifications of our previous procedure

[45,49]. In brief, 100 μL 0.2 *M* triethylamine buffer (pH 7) was added to 0.45 mL serum or plasma followed by incubation with 40 μL β-glucuronidase and 40 μL arylsulfatase for 12–17 h at 37 °C. After addition of 20 μL formononetin (10 ppm in methanol) as internal standard, proteins were precipitated by vortexing with 0.45 mL acetonitrile for 1 min. Hydrolyzed analytes were then isolated by partitioning three times into 2 mL diethyl ether. The combined organic phases were evaporated under a stream of dry nitrogen, redissolved by vortexing with 0.1 mL methanol, diluted with 0.1 mL 0.2 *M* acetate buffer (pH 5), and 20 μL were injected into the LC system.

Unconjugated analytes were determined without enzymatic hydrolysis by straight extraction with diethyl ether or ethylacetate, in essence omitting incubation with β-glucuronidase and arylsulfatase. Unconjugated isoflavonoid levels in plasma or serum were only 1–3% of total concentrations (conjugated plus unconjugated), which is in excellent agreement with earlier reports [40,50,51].

2.4. LC–PDA–ESI–MS analysis

LC–PDA–ESI–MS analysis was performed using a HydroBond PS (100×3.0 mm, 5 μm) C_{18} reversed-phase column coupled to a HydroBond PS C_{18} (25×3.2 mm, 5 μm) direct-connect guard column with a mobile phase flow-rate of 0.25 mL per minute.

Mobile phase A (containing acid): methanol–acetonitrile–0.5% aqueous acetic acid from 20:20:60 linearly to 35:35:30 in 13 min followed by holding at 35:35:30 for 1 min, changing linearly to 45:45:10 in 1 min followed by holding there for 4 min, and changing linearly to 20:20:60 in 1 min with equilibration for 5 min before subsequent injection.

Mobile phase B (containing no acid): methanol–acetonitrile–water from 20:20:60 linearly to 35:35:30 in 13 min, changing linearly to 45:45:10 in 1 min followed by holding at this mixture for 4 min, then changing linearly to 20:20:60 in 1 min with equilibration at this ratio for 6 min before subsequent injection.

UV detection was performed between 220 and 400 nm [52] followed by mass spectrometric measurements on-line in the negative mode after electrospray ionization with mass screening covering the range 180–350 amu. The capillary temperature was held at 260 °C, and sheath nitrogen gas flow and auxiliary nitrogen gas flow was set at 90 and 30 arbitrary units, corresponding to approximately 90 and 10 p.s.i., respectively. ESI source needle voltage was set at 4.6 kV, leading to an average current of 80 μA, and capillary voltage was set at −8 V. The divert valve was programmed to allow flow into the mass spectrometer during analyte elution (approximately from 4 to 17 min of the HPLC run time). Individual analytes were monitored by screening M−1 masses (mass of molecular ion minus proton $[M-H]^-$) of parent ions alone or in combination with the most predominant first-generation product ions (cut off if abundance <5%) applying collision energies of 40–42% corresponding to 2.0–2.1 V of resonance excitation radio frequency voltage (see Table 1). Mass widths of 1–2 amu were applied during acquisitions in order to include diagnostic ions in case of slight mass spectrometry measurement inaccuracies. The following ions and transitions were monitored: daidzein at $M-1=m/z$ 253.1; dihydrodaidzein at $M-1=m/z$ 255.1; glycitein at $M-1=m/z$ 283.1 and first-generation product mass of m/z 268.3; genistein at $M-1=m/z$ 269.1 and first-generation product masses of m/z 201.4 and 225.5; dihydrogenistein at $M-1=m/z$ and first-generation product mass of m/z 165.3; *O*-desmethylangolensin at $M-1=m/z$ 257.1 and first-generation product mass of m/z 239.3; formononetin (internal standard) at $M-1=m/z$ 267.1 and first-generation product mass of m/z 252.3; hesperetin at $M-1=m/z$ 301.1 and first-generation product masses of m/z 283.3, 258.1, and 242.4; naringenin at $M-1=m/z$ 271.1 and first-generation product masses of m/z 177.4 and 151.2; enterodiol at $M-1=m/z$ 301.2 and first-generation product masses of m/z 283.3 and 253.4; enterolactone at $M-1=m/z$ 297.1 and first-generation product masses of m/z 253.4 and 189.4; quercetin at $M-1=m/z$ 301.1 and first-generation product masses of m/z 151.3 and 179.3; equol at $M-1=m/z$ 241.1; and taxifolin (internal standard) at $M-1=m/z$ 303.1. Quantitation was performed on ions described in Table 1. Limit of quantitation, precision,

Table 1
Masses and absorbance data of analytes used for identification and quantitation

Analyte	Code	Nuclid mass	Full scan masses[a]	Masses after CID[a,b]	λ (ε)[c]	Ref.[d]
Enterodiol	ED	302.1518	M−1	241.7 (14%), **253.4** (100%), **283.3** (49%)	275–281 (5012)	[e]
Enterolactone	EL	298.1205	M−1	**189.4** (38%), **253.4** (100%)	281 (4529)	[e,f]
Naringenin	NE	272.0685	M−1	**151.2** (100%), **177.4** (30%)	290 (17 600)	[55]
Hesperetin	HT	302.0790	M−1	151.5 (3%), **242.4** (100%), **258.1** (27%), **283.3** (83%)	289 (18 621)	[55]
Quercetin	Q	302.0427	M−1	**151.3** (45%), **179.3** (100%)	373 (20 892)	[56]
Daidzein	DE	254.0579	**M−1**	**253.7** (100%)	250 (20 893)	[57]
Dihydro-daidzein	DHDE	256.0736	**M−1**	223.3 (5%), **255.5** (100%),	277 (13 600)	[58]
Glycitein	GLYE	284.0685	M−1	**286.3** (100%)	256 (22 387)	[59]
Genistein	GE	270.0528	**M−1**	**201.4** (7%), **225.4** 9%), 241.4 (5%), **269.4** (100%)	263 (37 154)	[57]
Dihydro-genistein	DHGE	272.0685	M−1	**165.3** (100%), 225.7 (4%)	290 (18 300)	[58]
O-Desmethyl-angolensin	DMA	258.0892	M−1	**239.3** (100%)	280 (12 023)	[g]
Formononetin (internal standard)	FOR	268.0736	M−1	**252.3** (100%)	256 (29 512)	[57]
Taxifolin (internal standard)	TA	304.0583	**M−1**	**303.1** (100%)	290 (19 953)	[60]
Equol	EQ	242.0943	**M−1**	**241.2** (100%)	281 (6761)	[g]

[a] Observed by ESI and monitoring in negative mode; in bold letters if mass was used for quantitation by SRM.
[b] Abundance in parentheses.
[c] λ, absorption maximum (nm); ε, molar extinction coefficient measured in alcohol [55].
[d] References used for molar extinction coefficients.
[e] Mean of data obtained by private communications with Drs. Adlercreutz, University of Helsinki, Finland, and Metzler, University of Karlsruhe, Germany.
[f] Metzler, University of Karlsruhe, Germany, private communication, 1998.
[g] Wähälä, University of Helsinki, Finland, private communication, 1994.

and spiking recovery of this method for urinary and plasma analysis are shown in Tables 2–4.

2.5. Intra-individual variability study

A pilot study for the determination of consistency between three baseline measurements of phytoestrogens was conducted with 20 healthy volunteers in Hawai'i (11 women and nine men in the age range 30–60 years). Each subject continued on her or his habitual Western-style diet and provided overnight fasting blood and overnight urine samples on 3 working days, about 8 days apart, avoiding collection on the same day of the week. Blood was collected in green-top sodium-heparin vacutainers which were immediately processed followed by storage of plas-

Table 2
Assay repeatability for urine and plasma

	DE	GLYE	GE	DMA	NE	HT	ED	EL	DHDE[a]	DHGE[a]	Q[a]
Urine											
Mean level[b] (n*M*)	4714	587	3801	13	408	104	48	366	2394	8324	219
Intra-C.V. (%)	7	5	6	17	7	15	17	9	1	12	7
Inter-C.V. (%)	7	9	12	49	7	21	8	9	1	19	12
Plasma											
Mean level[c] (n*M*)	322	38	409	4	162	30	1	19			
Inter-C.V. (%)	11	23	24	70	14	34	38	39			
Mean level[c] (n*M*)	553	76	803	16	162	189	3	34			
Inter-C.V. (%)	12	8	8	23	6	6	48	17			
Mean level[d] (n*M*)	586	57	813	22							
Intra-C.V. (%)	0	13	12	14							
Inter-C.V. (%)	11	15	18	19							

Inter-C.V., inter-assay coefficient of variation; intra-C.V., intra-assay coefficient of variation.
[a] Not included in plasma analysis.
[b] Mean of 41 duplicate samples.
[c] Mean of 12 duplicate samples.
[d] Mean of five duplicate samples.

ma at −70 °C as described previously [45]. Urine specimens were preserved with boric and ascorbic acid and processed as described previously [46]. The procedures of this protocol were approved by the University of Hawai'i Committee on Human Subjects, and informed consent was obtained from all participants.

2.6. A feeding study with onions

A feeding study with onions was performed among six volunteers (four men, two women), aged 24–65 years, who were recruited for a 5-week randomized, cross-over study. All subject were smokers and in good general health. The feeding study consisted of 1 week on a low fruit-and-vegetable diet (basal diet), followed by 1 week on the same diet to which either 75 or 150 g/d of cooked onions was added. After a 1-week wash-out period during which subjects went back on their own regular diet, participants received the basal diet for a week followed by a week of basal diet plus the other dose of onions. Subjects were instructed not to eat anything outside the study diet. Twelve-hour urine collections were obtained at baseline and at the end of every study week. Urine specimens were kept refrigerated until processing. The procedures of this protocol were approved by the University of Hawai'i Committee on Human Subjects, and consent was obtained from all participants. Changes in quercetin excretion rates were calculated for statistical significance with the paired *t*-test since each subject served as his/her own control. Total phenol levels were determined colorimetrically with the Folin–Ciocalteu reagent as reported previously [48].

2.7. Statistical calculations

The weighted kappa κ_w is the percent agreement in a cross-tabulation of the value from one measurement (here, one urine collection) and the value from a second measurement, corrected for chance agreement and weighted by the level of agreement [53]. The disagreement weights used were $(i-j)^2/(1-m)^2$, where i is the column category, j is the row category, and m is the total number of categories. Fleiss and Cohen [54] show that when the disagreement weights are based on the squared distance $(i-j)^2$, κ_w is asymptotically equivalent to the intraclass correlation coefficient with categories assigned integer scaling (e.g., 1, 2, 3). The distribution of each flavonoid was divided into tertiles based on the 60 (20 subjects×3 samples) urine specimens; tertiles were used to ensure a large enough number in the

Table 3
Spiking recoveries from urine and plasma

	DE	GLYE	GE	DMA	NE	HT	ED	EL	DHDE[a]	DHGE[a]	Q[a]
Urine											
Urine level (n*M*)	3747	958	1116	294	15 868	142	240	3016	2394	8324	7060
Urine amount (nmol)	1.874	0.479	0.558	0.147	7.934	0.071	0.120	1.508	1.197	4.162	3.530
Spike level (n*M*)	8110	5566	5309	4991	14932	1043	1289	3439	2500	2500	3785
Spike amount (nmol)	4.055	2.783	2.654	2.496	7.466	0.521	0.644	1.719	1.250	1.250	1.892
Percent recovery	101	105	93	108	75	79	87	80	96	118	85
Urine level (n*M*)	400	108	69	27	3129	21	22	630	259	30	597
Urine amount (nmol)	0.200	0.054	0.034	0.013	1.564	0.011	0.011	0.315	0.130	0.015	0.298
Spike level (n*M*)	3567	4271	5152	4559	3863	1154	1239	1703	1250	1250	3230
Spike amount (nmol)	1.783	2.135	2.576	2.280	1.931	0.577	0.620	0.852	0.625	0.625	1.615
Percent recovery	73	95	112	104	63	99	101%	85	107	93	98
Urine level (n*M*)	3198	610	464	123	2508	125	343				
Urine amount (nmol)	1.599	0.305	0.232	0.061	1.254	0.062	0.171				
Spike level (n*M*)	2500	2500	2500	2500	500	500	500				
Spike amount (nmol)	1.250	1.250	1.250	1.250	0.250	0.250	0.250				
Percent recovery	108	60	89	86	65	92	80				
Plasma											
Plasma level (n*M*)	58	28	26	20	77	95	212	478			
Plasma amount (nmol)	0.029	0.014	0.013	0.010	0.038	0.048	0.038	0.048			
Spike level (n*M*)	27	24	20	20	24	37	50	250			
Spike amount (nmol)	0.014	0.012	0.010	0.010	0.012	0.018	0.012	0.018			
Percent recovery	113	114	103	100	80	84	87	91			
Plasma level (n*M*)	1675	173	2526	29							
Plasma amount (nmol)	0.838	0.086	1.263	0.015							
Spike level (n*M*)	24	24	24	24							
Spike amount (nmol)	0.012	0.012	0.012	0.012							
Percent recovery	91	96	113	117							
Plasma level (n*M*)	496	660	522	478							
Plasma amount (nmol)	0.248	0.330	0.261	0.239							
Spike level (n*M*)	238	238	238	238							
Spike amount (nmol)	0.119	0.119	0.119	0.119							
Percent recovery	79	106	84	77							

[a] Not included in plasma analysis.

cells. A weighted kappa was then computed for the 3×3 table comparing each pair of measurements (collection 1 vs. collection 2, 1 vs. 3 and 2 vs. 3). We report the average of these three kappa statistics. In general, the three κ_w for each flavonoid were similar, and there was no discernable pattern of agreement (e.g., the 1 vs. 3 κ_w was not consistently lower than the others even though more time transpired between the collections). A kappa statistic of 0 indicates that there is no agreement beyond that of chance between the measurements, while a kappa statistic of 1 indicates perfect agreement.

3. Results and discussion

3.1. Chromatography and mass spectrometry

We found greatly improved chromatographic performance for the analytes of interest by using a stationary C_{18} reversed phase with polar endcapping in the alkyl chain, such as in Hydrobond PS columns, due to the robustness of the stationary material at low pH and the resistance of matrix collapse with aqueous mobile phases containing low or no organic modifier (Figs. 1 and 2, upper panel). Small addition

Table 4
Limit of quantitation in urine and plasma[a]

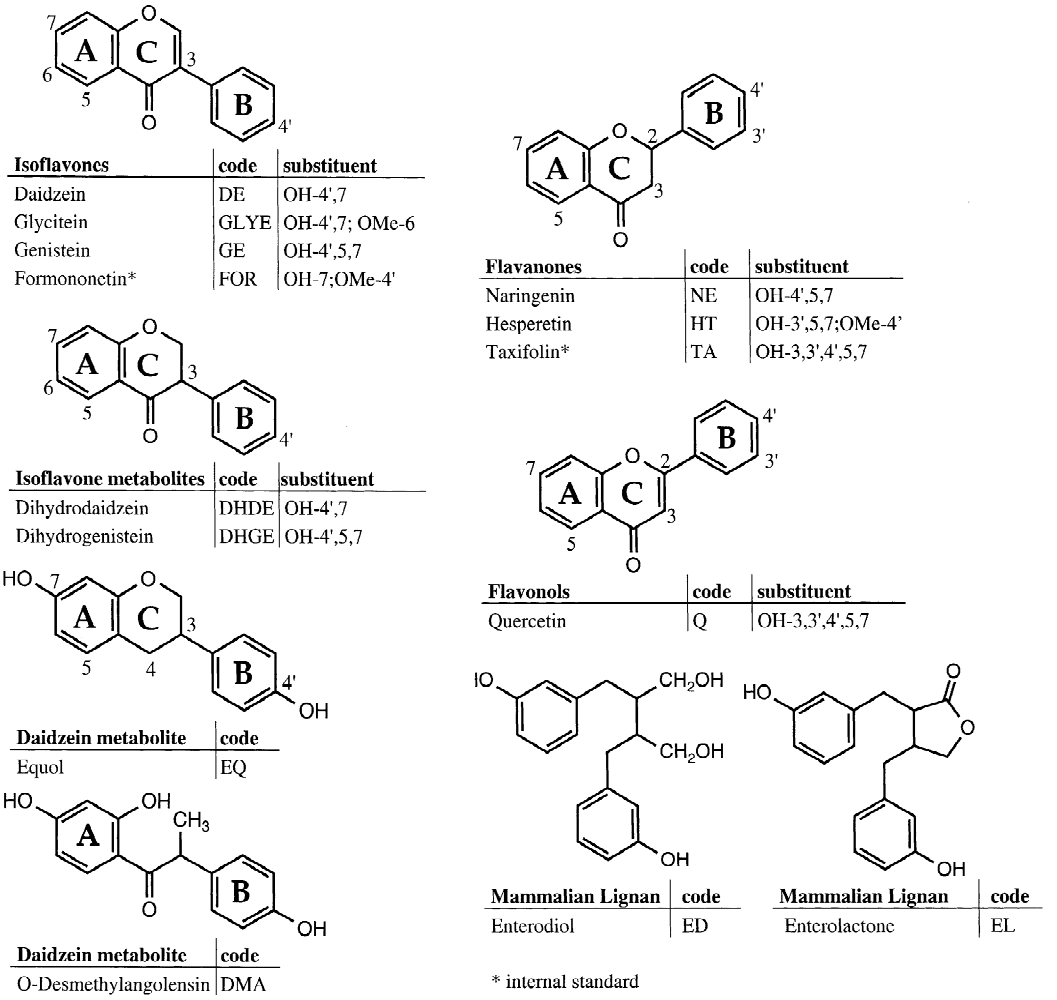
	DE	GLYE	GE	DMA	NE	HT	ED	EL	DHDE[b]	DHGE[b]	Q[b]
Urine[c]											
n*M*	27	2	4	3	<1	6	2	2	39	2	33
fmol	544	30	84	55	4	116	44	38	786	36	660
pg	138	9	23	14	1	35	13	11	201	10	200
Plasma[d]											
n*M*	29	1	2	3	<1	<1	1	<1			
fmol	583	16	42	52	4	12	29	11			
pg	148	4	11	13	1	4	9	3			
n*M*	20	1	3	2	1	2	2	2			
fmol	400	14	50	40	4	12	35	11			
pg	102	4	14	10	1	4	11	3			

[a] Values given as mean in two studies on plasma levels and one study on urinary excretion rate using 20 μL HPLC injections.
[b] Not included in plasma analysis.
[c] Acetic acid in mobile phase; average of six samples.
[d] No acetic acid in mobile phase.

of acetic acid to the mobile phase resulted in slightly less mass detection sensitivity using electrospray ionization in the negative mode due to suppression of deprotonation leading to a decrease in anionic phenolate species in solution. However, a vastly improved chromatographic separation of the analytes of interest was achieved with sharper signals and higher peak symmetry by small acid addition. This allowed mass screening acquisitions with a higher scan rate (scan events per second per analyte), leading to higher selectivity and lowering the baseline noise and limit of quantitation. A similar finding regarding an overall improvement of LC–MS performance by lowering the pH of the HPLC mobile phase for slightly acidic analytes has been reported previously [61]. Monitoring positive ions was found to be inferior relative to negative mode even with acid-containing mobile phases due to lower selectivity and high background noise. We therefore chose monitoring in negative mode using a mobile phase without acetic acid for applications with a limited number of analytes requiring high sensitivity, for example the analysis of isoflavones in plasma. A mobile phase containing 0.5% acetic acid was selected for complex mixtures with numerous analytes, for example the analysis of isoflavones, lignans and flavonoids in urine. With the established MS conditions using ESI in negative mode (see Experimental) we observed from all analytes predominant deprotonated parent ions (M−1). With the existing ion trap technology, most selective fragmentation of parent analyte ions was observed when 40–42% collision-induced dissociation (CID) energy was applied. Quantitation was performed under these CID conditions on parent ions alone if no diagnostic product ions could be obtained (daidzein, dihydrodaidzein, equol). All other analytes formed M−1 ions and diagnostic product signals under these conditions. Distinct fragmentation under the selected ionization and CID conditions occurred generally under loss of unspecific moieties such as methyl groups, water, or CO_2 (15, 18, 44 amu) or, for example, for naringenin, quercetin and enterolactone, under the formation of characteristic flavonoid and lignan ions (Fig. 3). Tentative structures of fragments formed according to well-documented pathways are shown in Fig. 3 [62]. Pathway ''a'' yields a retro-Diels–Alder (RDA) product giving information of the substitution pattern in ring A of the flavonoid. Under loss of phenyl moiety B pathway ''b'' leads to a chromone (flavylium analogue), which gives additional information on the substitution at the flavonoid C-3 position. Enterolactone loses a kresol moiety to form a α- and β-substituted γ-lactone (Fig. 3b).

3.2. Quantitation

Quantitation was performed by selected reaction monitoring (SRM), i.e. by monitoring product frag-



Isoflavones	code	substituent
Daidzein	DE	OH-4',7
Glycitein	GLYE	OH-4',7; OMe-6
Genistein	GE	OH-4',5,7
Formononetin*	FOR	OH-7;OMe-4'

Isoflavone metabolites	code	substituent
Dihydrodaidzein	DHDE	OH-4',7
Dihydrogenistein	DHGE	OH-4',5,7

Daidzein metabolite	code
Equol	EQ

Daidzein metabolite	code
O-Desmethylangolensin	DMA

Flavanones	code	substituent
Naringenin	NE	OH-4',5,7
Hesperetin	HT	OH-3',5,7;OMe-4'
Taxifolin*	TA	OH-3,3',4',5,7

Flavonols	code	substituent
Quercetin	Q	OH-3,3',4',5,7

Mammalian Lignan	code
Enterodiol	ED

Mammalian Lignan	code
Enterolactone	EL

* internal standard

Fig. 1. Molecular structures of analytes.

ments alone or in combination with the parent ion if that parent ion still had significant abundance after the CID experiment (genistein, equol). SRM mode is an extremely helpful tool for highly selective and sensitive quantitation of trace components in complex matrices. Similar to selected ion monitoring (SIM) SRM is a feature of ion trap technology and similar to real MS–MS experiments. SRM also monitors a limited number of preselected product masses. In contrast to SIM, SRM is much more selective because it limits the product ions to exclusively those deriving from preselected precursor ions, in our case the parent M−1 masses. This feature of LC–MS is highly advocated in order to avoid misidentification [39,44]. We followed this recommendation by quantitating all analytes on product fragments derived from their M−1 parent ions. Only equol, daidzein and dihydrodaidzein did not fragment and were therefore quantitated using the parent M−1 ion. Data acquisition of the non-fragmenting compounds daidzein and dihydrodaidzein with SIM did not result in a better limit of quantitation compared to full scan MS followed by extraction of M−1 signals. SIM was therefore not applied. This resulted in a lower number of MS experiments to be conducted during elution of peaks of interest, which was highly desirable due to the limitation on the number of scan events that can be

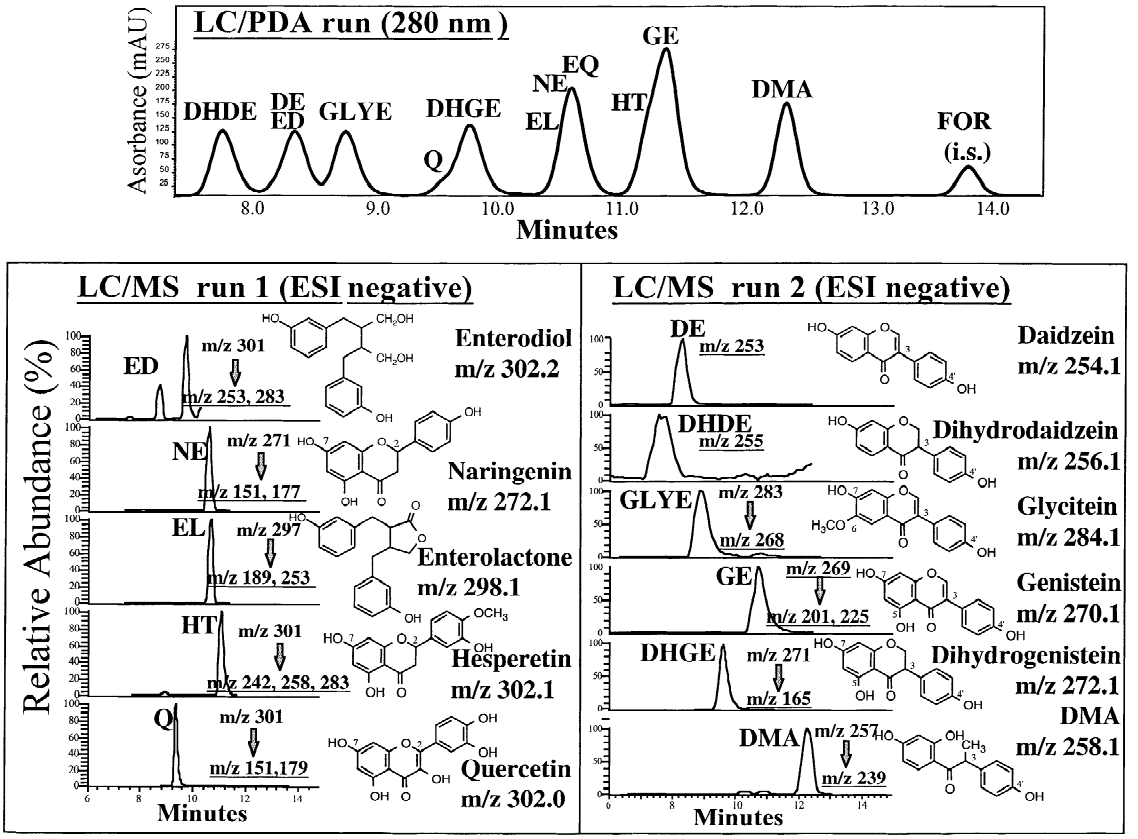


Fig. 2. Traces after LC separation using acid-containing mobile phase by PDA monitoring at 280 nm (upper panel) and simultaneously by MS analysis using SRM in negative mode after ESI (lower panel). Upper panel: some analytes coelute and cannot be quantitated by PDA monitoring. Lower panel: SRM traces obtained by monitoring analyte-specific fragments after applying collision energies of 40–42% (reaction pattern shown by arrows). Exact molecular nuclid masses of each analyte are shown to first decimal place. Diagnostic fragments from parent ions are shown by arrows. Quantitation was performed on the ions underlined: parent ions were used alone if no diagnostic daughter ions could be obtained by CID (daidzein, dihydro daidzein); for all other analytes, parent ions produced diagnostic daughter ions and quantitation was performed in these cases on these product masses alone or in combination with significantly abundant parent ions (genistein). i.s., internal standard.

performed within a time segment in which compounds elute. For high analyte levels, quantitation by PDA showed excellent correlations with that by MS. However, at low levels, PDA detection was inferior to MS measurement due to the high limit of quantitations and interference of co-eluting compound. More flavonoid agents, for example the flavones apigenin and luteolin or the flavonols myricetin and kämpferol [41], can be included in this assay if desired. Currently, the low dietary exposure to these compounds [63] does not warrant inclusion in the analysis of biological matrices for epidemiologic studies. Simultaneously to SRM we also monitored the mass range 150–600 amu during the entire MS run, which allows the retrieval of analytes not considered in the original assay. Although not as sensitive as SRM this detection mode may become an important asset because information on analytes is acquired that was not included in the original study design. This may emerge to be important for long-term projects like large prospective epidemiologic studies which might last over 5–10 years or even longer because interest in certain analytes may arise at a very late stage of the study. With the data

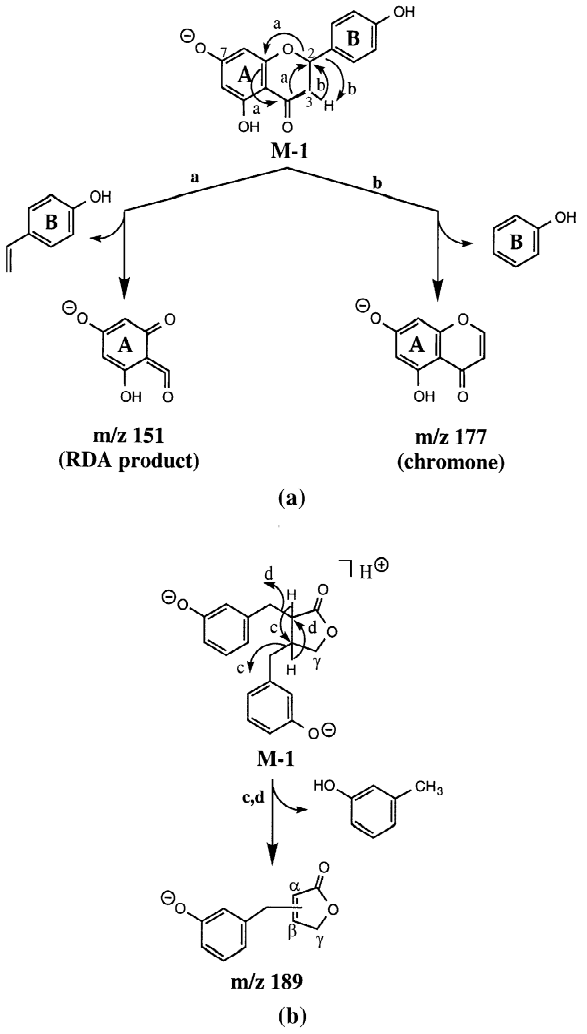


Fig. 3. (a) Tentative structure of diagnostically valuable flavonoid fragments under applied ESI conditions with monitoring in negative mode. Pathway a: retro-Diels–Alder (RDA) reaction. Pathway b: loss of phenol moiety B leads to a chromone. (b) Tentative structure of diagnostically valuable lignan fragments under applied ESI conditions with monitoring in negative mode. Pathway c and d: loss of kresol moiety leads to a α- and β-substituted γ-lactone ion at m/z 189, respectively.

obtained by ''full MS'', information on these analytes can be retrieved and included in the study after data acquisition.

Due to excessive data accumulation when all analytes were included in the assay we performed two chromatographic runs with discriminating mass screening of selected analyte groups. One run used mass-dependent tuning parameters for lignans and flavonoids (see run 1 in Fig. 2) and the other mass-dependent tuning parameters for isoflavonoids (run 2 in Fig. 2). This approach ensured sensitive and selective quantitation for all analytes of interest. Equol responded with relatively low sensitivity under these mass spectrometry conditions, which is in agreement with earlier reports when LC–MS with heated nebulizer atmospheric pressure chemical ionization was applied [44,64]. This was suggested to be due to the lack of a keto function in the molecule, thereby preventing ionization [64]. However, most recently we were able to include equol in this assay with satisfactory sensitivity by applying Z-spray ionization and by increasing fragmentation energies.

3.3. Calibration

Calibration was performed as described previously [12] using molar extinction coefficients as listed in Table 1 for accurate determination of stock solutions. Calibration curves obtained after spiking standards to plasma or urine did not change significantly from those obtained by pure standards dissolved in methanol–water mixtures after adjusting for analyte levels contained in the matrix. Consequently, no matrix interference occurred during this assay. The established ESI–MS mode resulted in little within-day but considerable day-to-day response variation. For example, daidzein, glycitein, genistein and *O*-desmethylangolensin were observed to be detected with a coefficient of variation of 11, 8, 10, and 9%, respectively, on 5 different days. Therefore, calibration curves were newly generated on each day of analysis for each analyte to minimize inter-assay variability. Low within-day variability of the calibration curve was assured by runs of one or two concentrations of the calibration curve of each analyte. In addition, the internal standard in each sample and at least one external urine or plasma standard per day assured accuracy during analysis.

3.4. Validation

Validation of the established assay was performed by determining precision (Table 2), spiking recovery (Table 3) and limits of quantitation (Table 4) using urine and plasma specimen from volunteers or

participants of epidemiologic studies. After adjusting each analysis for the recovery of the internal standard (formononetin or taxifolin) all quality assurance criteria for a reliable assay were met. In particular, intra- and inter-assay precision values for all analytes built a sound foundation for accurate analyses as required for large epidemiologic studies. Limits of quantitation of pure standards were lower by a factor of 5–20 due to background noise of biological matrices which we observed in complex serum as present in dialysis patients. Assay interferences are commonly known from serum of dialysis patients due to the lack of kidney clearance and the inability of dialysis to entirely remove all waste products from blood [49]. Due to inter-individual variability in plasma specimen, validation data are given as a mean of samples measured in different epidemiologic studies. Data variations as shown in Tables 2–4 are in part due to the use of different HPLC elution systems with mobile phase A containing no acid while mobile phase B contained 0.5% aqueous acetic acid which led to differential MS responses as mentioned above. Dihydrodaidzein and dihydrogenistein as well as quercetin validation was performed exclusively for urinary analyses because urine is the preferred matrix for phytoestrogen measurements.

3.5. Temporal variation in phytoestrogens

Temporal variation in phytoestrogens was assessed in a pilot study with 20 healthy volunteers in Hawai'i providing three consecutive blood and urine specimens. We determined the degree of consistency or agreement between the three baseline measurements for individuals. Quercetin was not included in these calculations because the assay was not fine-tuned for sensitive quantitation of this analyte at the time of analysis. The intra-class correlation coefficient is a statistic that measures the percentage of variance accounted for by between-subject variability, based on the results of a variance components analysis [65]. The distributions of the urinary phytochemicals were highly skewed to the right, and transformation was not effective at meeting the analytic assumptions of variance components analysis. Therefore, we used weighted kappa statistics to measure agreement (Table 5, see Experimental). As noted in the Statistical calculations section, a kappa statistic of 0 indicates that there is no agreement beyond that of chance between the measurements, while a kappa statistic of 1 indicates perfect agreement. We found good agreement for DMA (0.50), enterodiol (0.52), enterolactone (0.74) and the sum of both lignans (0.64). Within-person variability is much larger than

Table 5
Degree of consistency between three measurements on baseline urinary excretion rates of isoflavonoids, flavonoids and mammalian lignans

Analyte	Weighted kappa[a]			Average
	1 vs. 2	1 vs. 3	2 vs. 3	
Daidzein	0.66	0.23	−0.03	0.29
Glycitein	0.46	0.37	0.18	0.34
Genistein	0.31	0.40	0.38	0.36
DMA	0.39	0.57	0.53	0.50
All soy isoflavonoids	0.64	0.38	0.18	0.40
Enterodiol	0.54	0.55	0.48	0.52
Enterolactone	0.67	0.74	0.82	0.74
Both lignans	0.64	0.60	0.70	0.64
Naringenin	−0.16	0.07	0.17	0.03
Hesperetin	0.18	0.33	0.34	0.28
Both citrus flavonoids	−0.08	0.16	0.25	0.11
Quercetin	0.22	−0.26	−0.14	−0.06
Total phenols[b]	0.01	0.39	0.23	0.21

[a] The weighted kappa is the average of the three pairwise κ_w values comparing the three urine collections. It is the percent agreement (exact within tertile) minus the agreement expected by chance, and gives weight to partial disagreements: weights for agreement within same tertile = 1; weights for agreement at +1 tertile (e.g., tertile 1 and tertile 2) = 0.75; weights for agreement at +2 tertiles (e.g., tertile 1 and tertile 3) = 0.

[b] Determined colorimetrically with the Folin–Ciocalteu reagent [48].

between-person variability for daidzein (0.29), glycitein (0.34), genistein (0.36), all soy isoflavonoids (0.40) and hesperetin (0.28) in the population investigated. The agreement between collection days for total phenols (0.21) and naringenin (0.03) was poor. When the between-person variability dominates the total variance, one measurement can classify an individual. If the within-person variability is large compared to the between-person variability, one measurement becomes more unlikely to provide a good measure of a person's long-term diet. Also, large within-person variability in an exposure variable deleteriously affects the power of a study to find an association between the disease and the exposure. The agreement levels exhibited for DMA, enterodiol, enterolactone and the sum of both lignans indicate that there is substantial within-person variability (between 26 and 50% of the total). However, well-known risk factors of disease have been shown to have similar agreement levels. For instance, the intra-class correlation coefficient for serum cholesterol measures has been found to be about 0.65 [66]. Agreement levels found for daidzein, glycitein, genistein, all soy isoflavonoids and hesperetin would seriously affect a study's power, requiring a very large sample size to mitigate the attenuation. Studies using these compounds should consider multiple specimen collections per person or a reproducibility study that will allow for the deattenuation of the association between disease and exposure.

3.6. Onion intervention study

Quercetin, although an ubiquitous dietary flavonoid [67], was also included in this assay due to its exceptionally high concentration in onions (100–800 ppm) [63,68–70] and the recent reports on its potential cancer preventive activity [28–33]. Despite recent findings of very rapid quercetin metabolism and degradation after onion intake by humans [71,72] we observed a significant increase of urinary quercetin excretion rates after intervention with two different onion doses (Fig. 4). When four men and two women, all smokers and in good general health, were fed in a cross-over design for 1 week a low fruit-and-vegetable diet (basal diet), followed by 1 week on this diet to which either 75 or 150 g/d of cooked onions was added, quercetin excretion rates in 12-h urine collections obtained at baseline and at the end of every study week were observed to increase significantly over rates at baseline or after a self-selected diet. Although we saw a 49% increase in urinary quercetin excretion rates after 150 g of onions per day versus 75 g per day, the difference

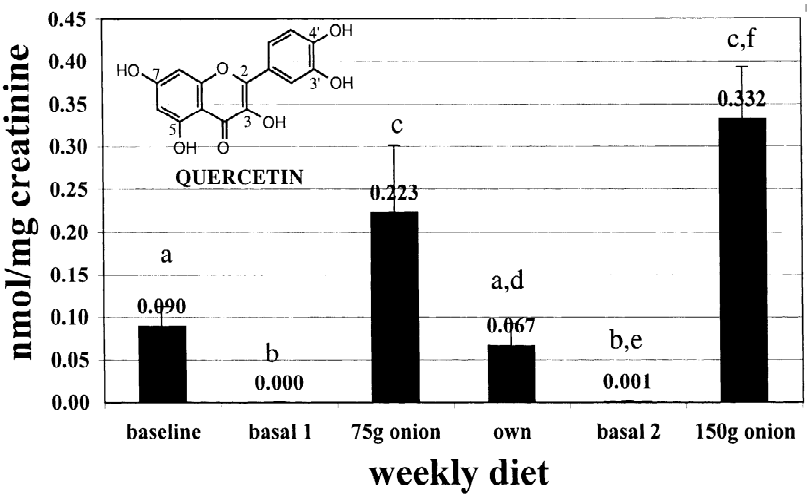


Fig. 4. Mean urinary quercetin excretion rates after onion intervention in six healthy subjects. After a self-selected diet (baseline), each subject consumed for 1 week in a cross-over design a basal diet low in phytochemicals (basal 1), then daily 75 or 150 g onion followed by a self-selected diet (own), then again a basal diet (basal 2), and finally 150 or 75 g onions, respectively. Urine was collected at the end of each week. Error bars indicate standard error. Columns with different letters are significantly different at $P \leq 0.05$.

did not reach statistical significance, probably due to the very small sample size. The very low excretion rates in the week following the onion doses indicate the very rapid half-life known for flavonoid agents [51,52,73]. Our finding of the presence of quercetin in urine after onion consumption is in good agreement with most recent discoveries on the occurrence of quercetin conjugates in human plasma and, to a small extent, in human urine after onion intake [74–76] and shows a convincing dose–response.

3.7. Epidemiologic studies

This method is currently being applied in several ongoing epidemiologic and clinical trials. The most recent results could confirm our previous findings on the negative association between urinary isoflavone excretion and breast cancer risk in Chinese women [48] by increasing the sample size from 120 to over 500 and by the application of the present, much more dependable, assay [77].

4. Conclusions

The presented method determines the most predominant dietary phytoestrogens at affordable cost in human blood and urine with high speed, accuracy, precision and sensitivity suitable for epidemiologic and clinical studies aimed at assessing the role of these phytochemicals in health and disease. For these studies we recommend collection of multiple samples from one individual due to significant intra-individual variability. We conclude from our experience in dietary intervention studies that quercetin in urine can be used as a useful biomarker of onion intake.

5. Nomenclature

amu	atom mass units
CID	collision-induced dissociation
ECD	electrochemical detection
ESI	electrospray ionization
LC	liquid chromatography
M	mass of molecular ion
M−1	mass of molecular ion minus one mass unit
MS	mass spectrometry
PDA	photo-diode array detection
UV–Vis	ultra-violet–visible detection
SIM	selected ion monitoring
SRM	selected reaction monitoring

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