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High-performance liquid chromatographic assay of isoflavonoids and coumestrol from human urine☆

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Abstract

A rapid, sensitive and precise diode-array reversed-phase HPLC method was developed for human urine analysis of the most common dietary isoflavones daidzein, genistein, formononetin and biochanin-A, their mammalian metabolites equol and O-desmethylangolensin, and of coumestrol, another commonly occurring phytoestrogen. Analytes were isolated and concentrated by solid-phase extraction and separated by HPLC followed by identification through retention times and UV scans, and in the case of coumestrol additionally by fluorometric response. This method was applied to monitor changes in urinary excretion of these analytes after challenge with soybeans and was evaluated for precision and recovery of analytes.

1. Introduction

Isoflavonoids are plant products with a 3-phenyl chroman structure and are essentially confined to legumes [1]. They were classified as "phytoestrogens" [2] due to their estrogenic properties [3] discovered after isoflavonoids were found to have caused infertility syndromes in sheep [4,5] excessively grazing on forage plants [6]. Mammalian metabolic studies of the most common isoflavonoids (Fig. 1) revealed a fast conversion of dietary genistein (GE) to *p*-ethylphenol [5] whereas formononetin (FOR) and daidzein (DE) were metabolized by ruminal bacteria [7,8] to O-desmethylangolensin (DMA) [9] and equol (EQ) [5,10], and only the latter product was found to have estrogenic activities similar to the parent compounds [10].

The interest in isoflavones and their metabolites was sparked by reports about their potential cancer-protective properties [11–14] and about properties often connected with cancer prevention such as dose dependent estrogenic or antiestrogenic properties [2,13–17], antioxidant [18,19], radical scavenging [20], hypolipidemic and serum cholesterol lowering [21,22], antimutagenic [23], antiproliferative [24–26], differentiation inducing [27] and angiogenesis inhibiting [28] effects. In particular, the suggestive role of dietary isoflavones from soy products to reduce cancer risk [16,29,30], has increased the efforts to analyze these substances in foods [31–33] and in biological fluids [12,34–36]

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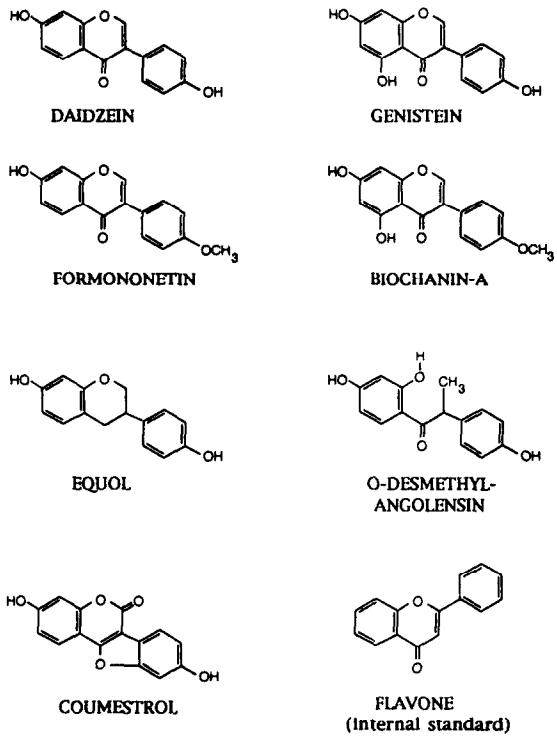

Fig. 1. Structures of analytes and internal standard.

Older methodology for the assay of isoflavonoids utilized spectrophotometric, fluorometric [37] or other techniques, such as paper chromatography [38] or TLC [39]. Due to the occurrence of phytoestrogens in mammals as predominantly glucuronide and sulfate conjugates [40,41], more modern techniques hydrolyzing biological samples prior to analysis in order to reduce the complexity of analytes have been introduced. Gas chromatographic (GC) methods enhancing sensitivity were introduced by Naim and coworkers [42], but required derivatization such as trimethylsilylation prior to measurement. The GC technique was improved by combining it with mass spectrometric (MS) detection [43], allowing peak identification. This method was applied to studies on isoflavonoid metabolism in humans [36] and led to the identification of EQ in human urine [44,45]. This methodology was finally refined by selected-ion monitoring resulting in the additional identification of DE in human urine [40,41] and by isotope dilution techniques resulting in simultaneous analyses of 3 lignans and 4 isoflavonoids including GE in human urine [34,46] and plasma [35].

Compared to GC–MS analysis high-performance liquid chromatography (HPLC) of isoflavonoids requires fewer steps for sample preparation, such as purification and derivatization, is less time consuming, less costly, demands less expensive instrumentation and has the advantage allowing the measurement of a variety of phytoestrogens including isoflavonoids, COM, aglycones and conjugated analytes in one run. The original HPLC method [47,48] was improved recently for food analyses using reversed-phase materials [31–33]. To our knowledge isoflavonoid analysis of biological fluids with HPLC was performed only in a limited number of exclusively animal studies without the use of internal standards [49] and without including conjugated analytes [50].

We present a fast, sensitive, reliable and precise diode-array reversed-phase HPLC method analyzing the most common dietary isoflavones DE, GE, FOR and B-A [1] and their mammalian metabolites EQ, DMA, and of COM, another commonly occurring phytoestrogen [2] in human urine using flavone as internal standard (Fig. 1). Urine samples were purified and concentrated by reversed-phase SPE followed by hydrolysis and analysis of aglycones by HPLC including peak identification by UV scans and fluorometric response. We evaluated the proposed procedure for hydrolysis and extraction efficiency, and for precision and spiking recovery.

This method was applied to monitor changes in urinary excretion of these analytes over time in one individual during four days and in 11 individuals during two consecutive nights after challenge with 5–20 g and 44–96 g roasted soybeans, respectively. Additionally, this method was evaluated for the feasibility of using urinary isoflavonoids as biomarkers for soy consumption in epidemiologic trials aimed at assessing the cancer preventive effects of soy foods.

2. Experimental

2.1. Apparatus

HPLC analyses were carried out on a system Gold chromatograph with an autosampler Model 507 and a dual-wavelength diode-array detector Model 168 (all units from Beckman; Fullerton, CA, USA) and a fluorescence detector Model FD100 (GTI/SpectroVision; Concord, MA, USA). Optical density readings were obtained from a DU-62 spectrophotometer (Beckman). Evaporation was performed with a Savant AS 160 Speed-Vac (Farmingdale, NY, USA).

2.2. Chemicals

Methanol, hydrochloric acid, acetic acid, 96% ethanol, dimethylsulfoxide (DMSO) and all solvents used for HPLC and optical density readings were analytical grade or HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Butylated hydroxytoluene (BHT), glucosidase (isolated from almonds), sodium acetate, biochanin-A (B-A) and glucuronidase/sulfatase (isolated from *Helix pomatia* type HP-2S) were purchased from Sigma (St. Louis, MO, USA). DE, FOR and GE were obtained from ICN (Costa Mesa, CA, USA) and flavone from Aldrich (Milwaukee, WI, USA). EQ and DMA were a kind gift from Dr. H. Adlercreutz (University of Helsinki, Finland).

2.3. Standard solutions, calibration curves and calculation of phytoestrogen levels

Phytoestrogen stock solutions were prepared by dissolving the crystalline standards first in 20 μl of DMSO followed by addition of 96% ethanol to give 2–5 molar solutions. The purity of these solutions was checked by HPLC analysis with monitoring at the individual compound's absorption maximum. The purity (%) of the standard was calculated by dividing the peak area of the compound by all peak areas in the chromatogram and multiplying by 100 assuming that contaminants or by-products have the same light absorption properties as the standard. Compounds with less than 95% purity were discarded. The concentration of the stock solutions was determined by optical density readings at the wavelength with maximum absorption (λ_{max}) using molar extinction coefficients (ϵ) [51] after diluting the stock solutions to appropriate concentrations with 96% ethanol except for COM which was diluted with acetonitrile [52] with the following values ($\lambda_{max}(\epsilon)$): DE = 250 nm (20 893), GE = 263 nm (37 154), FOR = 256 nm (29 512), B-A = 263 nm (27 542), COM = 339 nm (22 300), EQ = 281 nm (6761) and DMA = 320 nm (7586). The final stock concentration of each individual standard was calculated using the optical density reading adjusted for the purity.

Concentrations of analytes from urine were calculated with area units obtained from HPLC analyses and the slope of the calibration curve and were expressed as nanomoles per hour (nmoles/h) after adjustment for time period between urine collection and previous void (h), urine volume (ml) and internal standard recovery.

2.4. Chromatographic conditions

For solid-phase extraction (SPE) C_{18} reversed-phase columns were obtained from PGC Scientific (Gaithersburg, MD, USA).

HPLC analyses were carried out on a NovaPak C_{18} (150 × 3.9 mm I.D.; 4 μm) reversed-phase column (Waters; Milford, MA, USA) coupled to an Adsorbosphere C_{18} (10 × 4.6 mm I.D.; 5 μm) direct-connect guard column (Alltech; Deerfield, IL, USA). Elution was performed at a flow-rate of 0.8 ml/min with the following step gradient: A = acetonitrile, B = acetic acid–water (10/90, v/v); 20% A in B (v/v) for 16 min, then 70% A for 14 min and again 20% A for 10 min. Analytes were monitored with a dual-channel diode-array detector at 260 nm during the entire HPLC run, at 280 nm during EQ elution and at 342 nm during COM elution. Observed peaks were scanned between 190 and 400 nm. The fluorescence detector was used with a 340-nm excitation filter and a 418-nm

emission filter to selectively monitor coumestrol (COM).

2.5. Collection and handling of urine

Urine samples were stored in disposable bottles containing 0.2–0.4 g boric acid and 0.1–0.3 g sodium ascorbate (depending on expected urine volumes) to control for bacterial contamination and for degradation of analytes. After mixing and volume determination each urine sample was transferred into five 25-ml disposable plastic tubes and stored between −20°C and −70°C until analyzed. Times of actual urine collection and previous void were recorded for adjustment purposes.

2.6. Extraction of urinary phytoestrogens

Frozen urine aliquots were equilibrated to room temperature, vortex-mixed and centrifuged at 850 *g* for 20 min. Aliquots of 20.00 ml of clear supernatant were mixed with 5.0 ml 0.2 *M* acetate buffer (pH 4) and 200 μl flavone (60 ppm in 96% ethanol) as internal standard and filtered through a C_{18} RP-SPE column preconditioned with 3 ml of methanol and 3 ml of acetate buffer. After the urine has completely passed through the column, the column was washed with 2 ml of acetate buffer and the phytoestrogens were eluted with 100% methanol into microcentrifuge tubes to give exactly 2.00 ml. A 100-μl volume of this eluate was kept in an insert of an amber HPLC vial at −20°C until HPLC analysis for unconjugated phytoestrogens was carried out.

2.7. Enzymatic hydrolysis of urinary phytoestrogens

The residual 1.9 ml SPE eluate was dried by Speed-Vac at room temperature and incubated for 24 h at 37°C after mixing thoroughly with 0.9 ml of a freshly prepared mixture of 10 ml 0.2 *M* acetate buffer (pH 4), 150 mg ascorbic acid and 500 μl glucuronidase/sulfatase (isolated from *Helix Pomatia*) [34]. The hydrolyzed samples (total of unconjugated and hydrolyzed conjugated phytoestrogens) were then mixed with 1.0 ml of 100% methanol and stored at −20°C. The frozen samples were equilibrated to room temperature, vortex-mixed and centrifuged at 850 *g* for 5 min prior to HPLC injection.

In a parallel experiment 200 μl of flavone (60 ppm in 96% ethanol) were mixed with 0.9 ml buffer and 0.9 ml of methanol in the same batch as the urine extractions and stored in an amber HPLC vial at −20°C. After equilibration to room temperature the standard was analyzed by HPLC in the same batch with the urine extracts for internal standard recovery calculation purposes.

3. Results and discussion

3.1. Sample preparation

Reversed-phase SPE used to purify and concentrate urine samples did not lead to analyte losses as recoveries were found to be between 98% and 101%. Because urinary phytoestrogens in biological samples occur predominantly as glucuronate and sulfate conjugates and mixtures thereof, urine samples were hydrolyzed to reduce the number of analytes and to include conjugates below detection limits [33]. Although isoflavonoids were shown to be very stable even after boiling in ethanol containing 2 *M* HCl [33] acid hydrolysis conditions were found to degrade EQ rapidly [53]. Therefore, hydrolysis with glucuronidase/sulfatase [34] was chosen although it required longer reaction times compared to acid hydrolysis (24 h vs. 9 h) and involved introduction of DE into samples, however in negligible amounts (7 pmoles/sample [34]). Enzymatic hydrolysis had the additional advantage of requiring less technician time and involving less hazardous procedures, compared to refluxing and handling hot hydrochloric acid [53]. Hydrolysates obtained after 24 h incubation at 37°C could be injected without further purification onto the HPLC system after mixing with 1 ml of methanol and centrifugation since co-extracted compounds in the crude mixture separated from the analytes by HPLC did not dam-

age the HPLC system as experienced by more than 250 injections producing highly reproducible results.

3.2. HPLC conditions

The conditions for a selective and efficient separation of all analytes were modified slightly from those we recently developed for food analyses [33] by eluting with a step gradient and starting at a lower acetonitrile concentration resulting in resolution of the EQ peak from the GE signal (Fig. 2). Monitoring was carried out at 260, 280 and 342 nm to achieve sensitive detection of all analytes, and COM was additionally monitored selectively by fluorescence detection sequential to UV detection.

3.3. Identification of analytes

All phytoestrogens analyzed by the proposed procedure were identified by comparing retention times and UV absorption patterns with authentic standards analyzed in the same batch and with reported UV data [1,51]. COM was identified by the ratio of the 342-nm to the 260-nm absorption response and additionally, with fluorescence response obtained with a 340-nm excitation filter and a 418-nm emission filter [52].

3.4. Internal standards

Degradation or loss during analyte work-up and measurement are a constant source of potential errors requiring internal standards for precise and accurate analyses [54]. Flavone was selected as internal standard in this study among several candidates such as *o*-hydroxyacetophenone, *o*-methoxy-acetophenone, propiophenone, butyrophenone and 4-chromanone due to its structural similarity with phytoestrogens, its chromatography avoiding interference with the analytes (Fig. 2) and due to its stability [33,53].

3.5. Detection limits, calibration, precision and recovery

Detection limits (Table 1) obtained from authentic standards range between 5 and 780 n*M* for a 20-μl injection and allow sensitive phytoestrogen quantitations. Lower detection limits apply to urine samples due to the 10–20 fold concentration achieved during sample preparation with SPE. Detection limits can be decreased further by increasing the injection volume and, for COM by using fluorescence detection at higher pH of the mobile phase since the maximum 436-nm emission intensity of the COM mono-anion occurs at pH 8 [52].

Calibration curves were obtained with extremely high linearity in the concentration range of interest from all analytes included in this assay as shown in Table 2.

Precision and spiking recoveries (Tables 3 and 4) were found to be within accepted limits for phytoestrogen analyses [34,49] and confirm the validity of the proposed procedure.

3.6. Isoflavonoid analysis of human urine before and after soybean challenge

Urinary excretion of isoflavonoids and COM was followed for three days with 24-h urine collection using the proposed method described above in one healthy male individual (Fig. 3) before and after challenge with 5, 10 and 20 g roasted soybeans at time 0 and 22 h and 57 h later equaling a dose of 16.7, 33.4, 66.7 μmoles DE and of 18.6, 37.2 and 74.5 μmoles GE [33]. Urinary levels of DE, GE, EQ and DMA increased rapidly after soybean consumption and decreased 16–24 h thereafter to levels slightly above baseline levels except for DMA which showed a 10–16 h delay of this pattern (Fig. 3B). This might be due to the longer reaction time needed for DE to be converted to DMA compared to EQ due to the required fission of the pyran moiety, and/or is due to the fact that DMA is formed from ruminal bacteria [9] located in the more distal digestive tract. The soy doses (gram of beans) used in this regime correlate excellently with the observed isoflavonoid

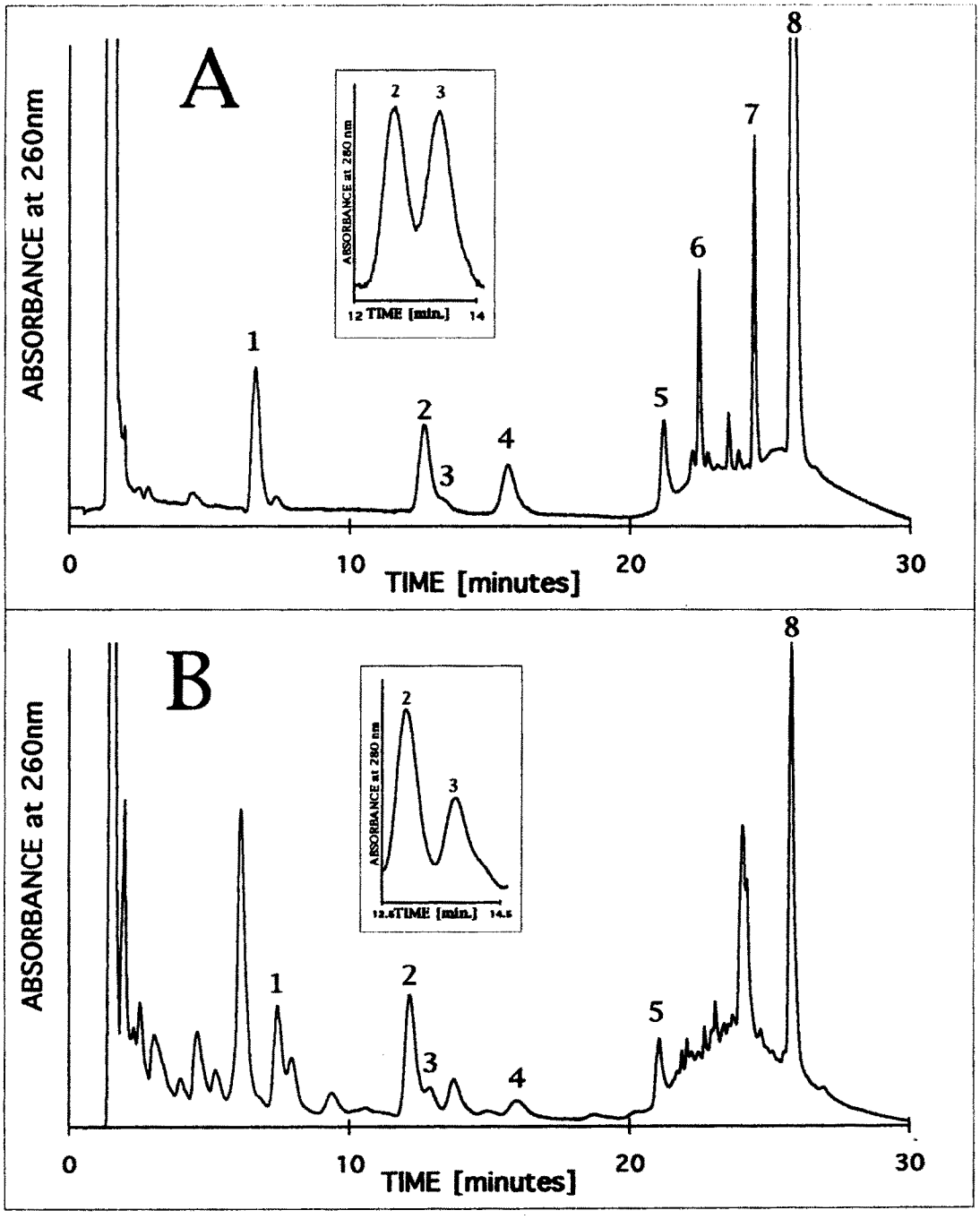

Fig. 2. HPLC trace of standards (A) and an urine extract (B) monitored at 260 nm and at 280 nm (insert) using a NovaPak C_{18} (150 × 3.9 mm I.D.; 4 μm) with an Adsorbosphere C_{18} guard column (10 × 4.6 mm I.D.; 5 μm). Elution was carried out at a flow-rate of 0.8 ml/min with the following step gradient : A = acetonitrile, B = acetic acid–water (10/90, v/v); 20% A in B (v/v) for 16 min and 70% A in B for 14 min followed by equilibration at 20% A for 10 min. Peak identification: 1 = daidzein, 2 = genistein, 3 = equol, 4 = coumestrol, 5 = O-desmethylangolensin, 6 = formononetin, 7 = biochanin-A, 8 = flavone (internal standard).

Table 1
Detection limits[a] of phytoestrogens analyzed by HPLC

Analyte	Detection limit		
	n*M*	ng/ml	pmol/h[b]
Daidzein	5.15	1.31	29.4
Genistein	8.75	2.37	50.0
Formononetin	7.25	1.95	41.4
Biochanin-A	13.00	3.70	74.3
Equol[c]	623.00	150.90	3560.0
O-Desmethylangolensin	780.10	201.47	4457.1
Coumestrol[d]	25.70	6.89	146.9

[a] Determined by peak height with a 20-μl HPLC injection at a signal-to-noise ratio of 5 and monitoring at 260 nm.
[b] Detection limit calculated for urine assuming 400 ml total volume collected during 7 h (20 ml extracted into 2.0 ml).
[c] Detection limit lower by a factor of 3.95 when monitored at 280 nm.
[d] Detection limit lower by a factor of 1.6 when monitored at 342 nm.

Table 2
Calibration parameters of analyzed isoflavonoids and coumestrol

Analyte	Concentration range (μM)	n[a]	Slope[c]	S.E.[b]	Intercept[c]	S.E.[b]	r^2 [d]
Daidzein	0.0–70.4	14	1.533	0.0043	−0.009	0.0817	0.999961
Genistein	0.0–41.7	10	0.802	0.0009	−0.013	0.0209	0.999996
Formononetin	0.0–34.5	10	0.735	0.0009	−0.018	0.0198	0.999995
Biochanin-A	0.0–52.9	10	1.051	0.0015	0.029	0.0342	0.999994
Equol	0.0–29.8	12	23.131	0.2589	0.419	0.1536	0.999499
O-Desmethylangoloensin	0.0–193.6	14	7.780	0.2632	0.707	2.7401	0.999183
Coumestrol	0.0–76.3	10	2.365	0.0062	−0.023	0.0900	0.999996

[a] Number of points.
[b] Standard error.
[c] Obtained by plotting concentration (μM) as a function of peak area.
[d] Coefficient of determination between concentration (μM) and peak area.

Table 3
Precision for phytoestrogen analysis from human urine

Sample	Daidzein[a] (nmol/h)	R.S.D. (%)	Genistein[a] (nmol/h)	R.S.D. (%)	Equol[a] (nmol/h)	R.S.D. (%)	O-Desmethyl-angolesin[a] (nmol/h)	R.S.D. (%)	Coumestrol[a] (nmol/h)	R.S.D. (%)
Within-assay (n = 3)										
1	2632.1	3.2	3309.5	4.0	1790.5	5.3	262.01	3.8	35.6	12.2
2	37.1	8.4	195.7	7.5	734.4	8.2	80.8	8.1	n.d.	
Between-assay (n = 2)										
3	2632.1	6.4	3309.5	3.9	1790.5	6.4	262.0	10.1	35.6	7.0
4	58.7	17.2	60.0	17.9	55.5	0.3	363.5	13.7	48.8	0.9

[a] Mean.

Table 4
Spiking recovery of phytoestrogens from human urine

	Daidzein	Genistein	Equol	O-Desmethyl-angolensin	Coumestrol
Sample 6					
Initial amount (nmol)	10.7	56.2	210.8	23.2	0.0
Spiked (nmol)	35.4	53.7	32.5	0.0	78.9
Recovery (%)	80.7	84.3	87.7		82.9
Sample 7					
Initial amount (nmol)	15.4	17.0	18.0	106.4	15.9
Spiked (nmol)	48.5	26.2	102.0	269.2	79.5
Recovery (%)	97.6	88.3	97.1	100.7	86.3
Spiked (nmol)	40.6	22.6	98.5	224.1	98.3
Recovery (%)	81.6	76.3	93.7	83.9	94.3

amounts (μmoles DE, GE, EQ or DMA) excreted in urine during the first 24 h after challenge (Table 5). DMA showed an even better correlation ($r = 0.9915$ vs. $r = 0.9782$) when 32-h instead of 24-h urinary amounts were plotted as a function of dose, again, due to its slower metabolism. A 24.8% urinary recovery was found for the supplementation of 247 μmoles DE/GE based on the sum of urinary amounts of DE/GE/EQ/DMA found during the 88-h monitoring period. Since analytes were still present at the end of this study (Fig. 3) the urinary recovery rate was estimated to be potentially 4–5% higher.

Free aglycones were found to occur in urine at very low levels (0.1–2% of total) and were not subsequently determined.

In a separate experiment the same analytes were monitored over 3 days in urine from 6 healthy women and 5 healthy men[1]. The individuals consumed 44–96 g roasted soybeans (147–321 μmoles DE and 164–357 μmoles GE [33]) during one day and urine was collected during the night before and during the first and second night after supplementation. Again, concentrations of DE, GE and EQ were found to be significantly increased from baseline levels in the urines collected during the first night after soybean consumption (Fig. 4). These elevated levels decreased in the second night urine samples down to levels marginally higher than baseline concentrations. DMA levels followed the same pattern without reaching significance in a paired t-test. However, levels were found to be significantly increased ($p < 0.0005$ by paired t-test) in both the second and first overnight samples based on the sum of excreted isoflavonoids.

Great variation between individuals was found for the urinary recovery of the examined soy isoflavones and their metabolites (Fig. 5A). Also, the phytoestrogen pattern differed greatly between individuals and changed within individuals from the first to the second overnight urine sample after challenge (Fig. 5B,C). This suggested significant inter-individual differences in absorption and a highly variable metabolism inter- and intra-individually over time. Interestingly, although all subjects excreted the dietary isoflavones DE and GE, some individuals did not excrete any metabolites while others eliminated greater or lesser amounts without any obvious correlation. This is probably due to variability between individuals in dietary habits leading to differences in the gut flora known to be responsible for the isoflavone metabolism [36]. The intra-individual differences of DMA formation can also be explained by the delayed formation of DMA as noted above. In any case,

[1] The values from one mole volunteer were excluded as this individual was known to have consumed soy products prior to the intervention trial.

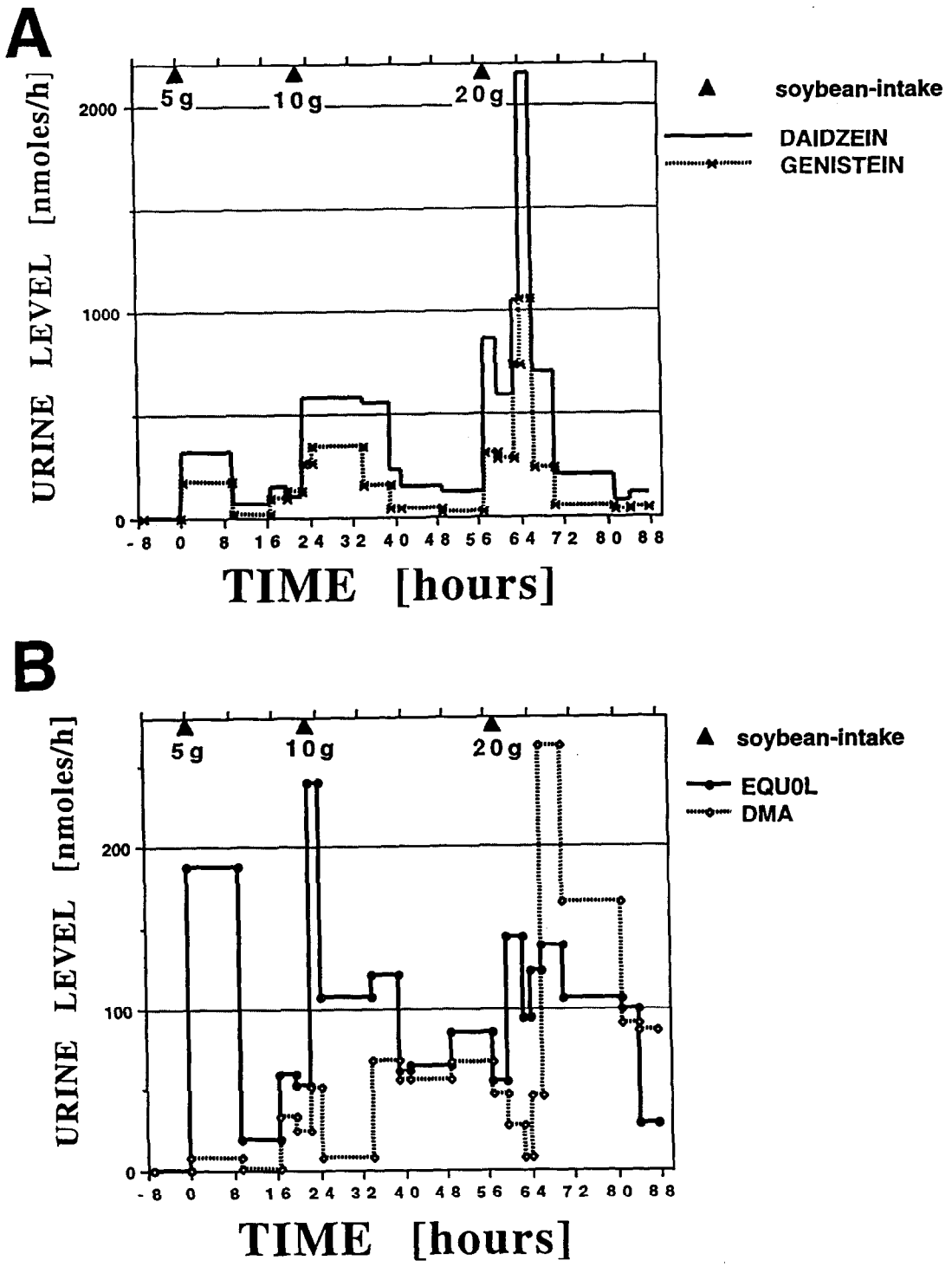

Fig. 3. Urinary isoflavonoid levels in one healthy male individual determined with proposed HPLC assay and monitored over a 88-h period after consumption of soybeans (5 g at time 0, 10 g 22 h later and 20 g 57 h later).

Table 5
Correlation between soybean dose[a] and urinary isoflavone amount[b] excreted in the first 24 h after challenge of one individual ($n = 4$)

	Slope[c]	S.E.[d]	Intercept[c]	S.E.[d]	r^e	p
Daidzein	3.6	0.5	4.8	5.4	0.9827	0.02
Genistein	317.9	71.3	729.7	816.7	0.9532	0.05
Equol	116.4	70.1	911.5	803.3	0.7612	0.24
DMA	165.8	24.9	−319.3	285.0	0.9782	0.02
DMA[f]	185.5	17.1	−203.6	195.8	0.9915	0.01
All analytes	1159.7	194	1727.9	2224.2	0.9704	0.03

[a] Gram soy beans consumed.
[b] Micromoles analyte excreted.
[c] Obtained by plotting urinary isoflavonoid amount in nmoles as a function of soybean dose in g.
[d] Standard error.
[e] Correlation coefficient.
[f] Urine amount excreted in the first 32 h after soybean challenge.

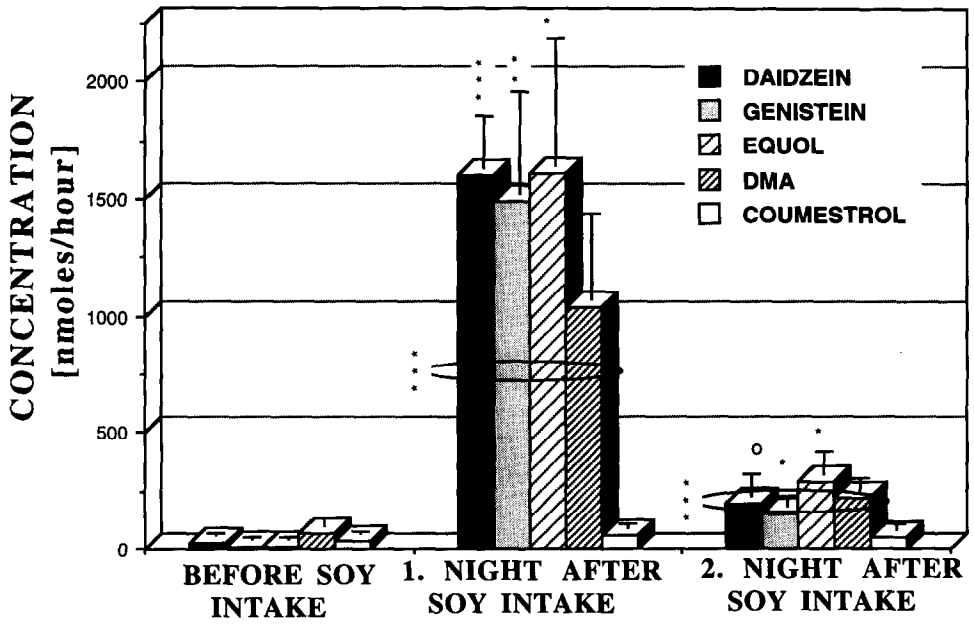

Fig. 4. Urinary phytoestrogen levels of 10 healthy subjects determined with proposed HPLC assay. The values represent mean levels (± standard error) of 6 females and 4 males in urine samples collected during the night before and during the first and second night after intake of 44–96 g soybeans (147–321 μmoles daidzein and 164–357 μmoles genistein). Significance of changes in urinary level after soybean intake were determined by a paired t-test comparing with levels before challenge and were noted with o for .05, * for .01, ** for .005 and with *** for .0005. The latter p-value was found for the first and the second night urine levels after challenge if the sum of DE, GE, EQ and DMA was used in the calculation (symbolized as circles).

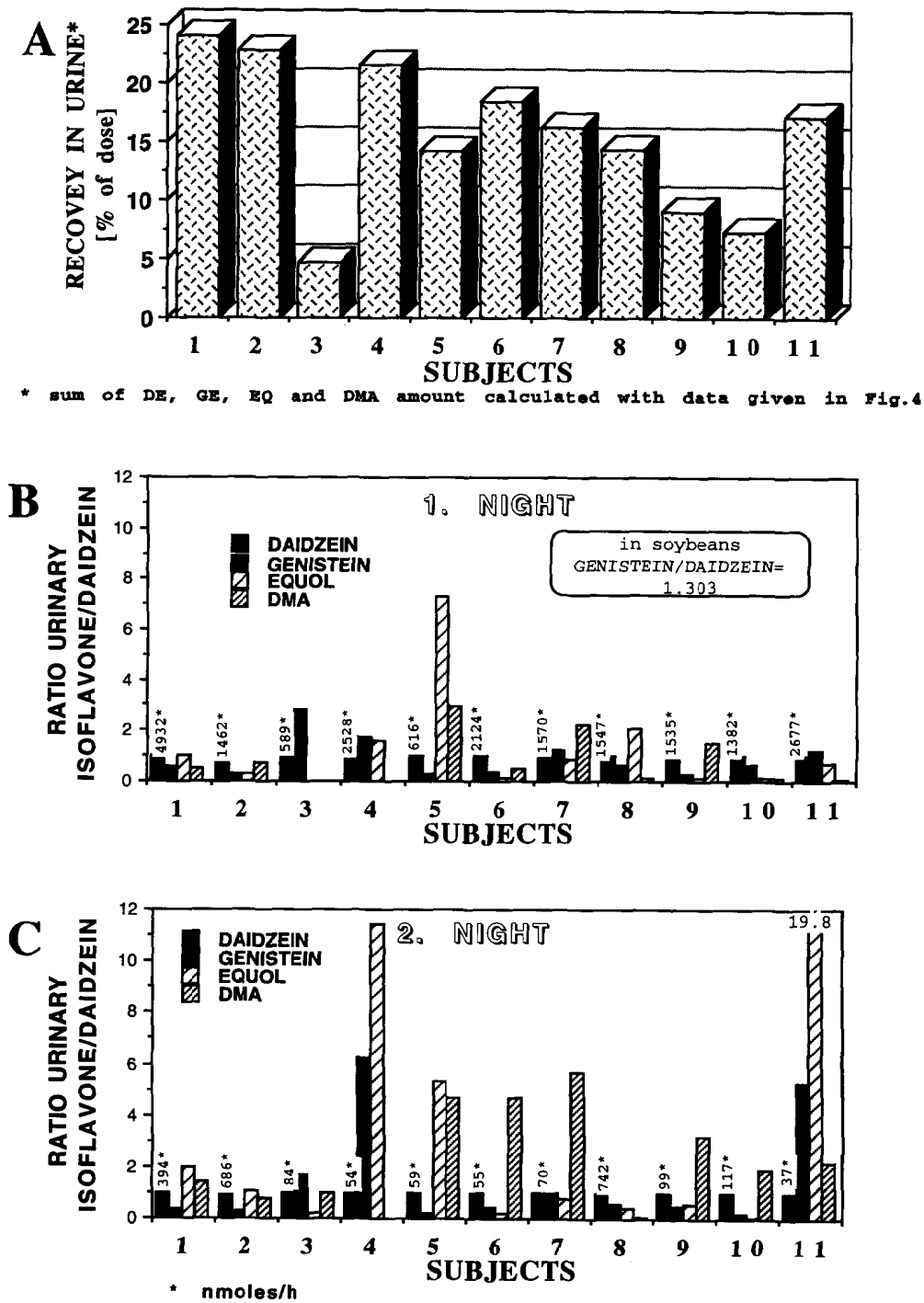

Fig. 5. Recovery (A) and excretion pattern found in urines of the first (B) and second (C) night after soybean challenge of 11 healthy individuals (6 females and 5 males). Great inter-individual differences were observed for total amounts and patterns of excreted analytes in urine which varied also intra-individually over time (see text for details).

these results indicate that levels of both dietary isoflavonoids and their metabolites must be determined in order to assess exposure to soy products or other phytoestrogen containing foods correctly. No dose response regarding urinary levels of analyzed phytoestrogens was observed in this intervention study which was probably due to supplementation above saturation level.

In a third application of the developed assay isoflavonoid and COM levels were monitored from 23 female subjects with overnight urine samples collected in 1986 as part of an epidemiologic study. Degradation of analytes is highly unlikely since the urine specimen were stored at −70°C and the analytes were shown to be very stable [33]. Food records indicated that the sub-population consuming tofu more frequently than once a week had significantly greater urinary levels of soy isoflavones and their metabolites compared to the subjects eating tofu less than once a week (Table 6). However, if the entire population was divided in four groups according to tofu intake the mean and median urinary isoflavonoid levels of the three quartiles with little intake were found to be very similar and lower by a factor of 2–15 compared to the quartile with highest intake. This observation might have been caused by urine samples of people with low intake not reflecting average isoflavonoid levels due to sampling at the "wrong" time, i.e. urine collection shortly after tofu consumption. The resulting misclassification could be corrected by combining three or, ideally, more urine samples in order to obtain urinary isoflavonoid levels accurately reflecting average intakes of these agents and the usual diet. The most feasible procedure for epidemiologic studies is probably a urine collection every other night over one week, since overnight urines contain concentrated levels of the analytes and are more accepted from participants resulting in higher compliance. Also, the analytes disappear from urine 48 h after intake and dietary habits should generally be represented over one week.

Table 6
Mean and median urine levels as a function of tofu consumption

Analyte	Tofu intake[a]	Urine concentration (nmol/h)	
		Mean	Median
Daidzein	>Once a week	179.8	38.3
	<Once a week	19.8	0.0
Genistein	>Once a week	104.8	18.4
	<Once a week	12.8	0.0
Equol	>Once a week	109.4	26.2
	<Once a week	26.8	2.5
DMA	>Once a week	11.6	22.0
	<Once a week	0.0	0.0
Sum Isoflavones	>Once a week	405.4	104.9
	<Once a week	76.8	14.1
Coumestrol	>Once a week	48.5	0.0
	<Once a week	34.1	31.0

[a] >Once a week group: $n = 7$ women; <once a week group: $n = 16$ women.

As expected, in all experiments described above, urinary levels of COM, FOR and B-A did not change after soy food consumption due to the absence of COM in soybeans [33] and since FOR and B-A occur only in few foods and only in minor amounts [33].

3.7. Correlation between urine and plasma levels

Cows challenged with DE and FOR rich forage showed increased plasma levels of these agents 1–3 h after ingestion and also of EQ, only 4 to 8 h delayed [49]. The very similar qualitative pattern we and others [46] found in human urines for isoflavones after challenge with soybeans rich in DE and GE indicates that these agents are absorbed quickly and eliminated in urine via partial metabolism to EQ and DMA. Additionally, excellent correlations between plasma and urine levels of DE, GE, EQ and DMA were found in 28 healthy Finnish women [35]. Therefore, isoflavonoid levels in urine reflect probably those in plasma and measuring urinary isoflavonoid levels should be a suitable

tool to determine exposure to these agents or to foods containing them.

However, absorption rates, biliary excretion and enterohepatic circulation of phytoestrogens remain to be studied in order to fully understand the fate of these agents in the humans system for accurate exposure determination.

4. Conclusions

We developed a rapid, precise and inexpensive HPLC assay for the analysis of DE, GE, EQ, DMA, FOR, B-A and COM from human urine with sufficient sensitivity to be used for the detection of pharmacologic or dietary doses of these analytes. Successful applications of this method revealed that all dietary analytes together with their most abundant metabolites must be determined in order to obtain reliable data reflecting exposure to these agents from food sources due to a highly variable inter-individual metabolism. Several urine samples from each individual in a population should be collected for accurate assessment of exposure to these analytes and their dietary sources. Compared to 24-h urine samples, overnight urine is more concentrated which facilitates the analysis and additionally, it is more compatible with epidemiologic studies as a result of easier implementation and higher compliance. Due to the fast metabolism of the analyzed phytoestrogens we recommend combination of three urine samples collected every other night from each subject to be tested in order to allow integration of an individual's phytoestrogen exposure over one week, a period generally reflecting usual dietary habits.

Due to the excellent agreement of the presented results with preliminary reports concerning urinary excretion of isoflavonoids after consumption of soy products using GC-FID [55] or GC–MS [56,57] the present assay is well suited for future epidemiologic trials assessing the role of isoflavonoids, phytoestrogens or soy consumption at preventing cancer or other diseases.

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