Phytoestrogens in Bovine Plasma and Milk – LC-MS/MS Analysis

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Abstract

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Phytoestrogens belong to a group of polyphenolic plant metabolites which induce biological responses, based on their structural similarity to 17β-estradiol. In order to investigate the relationship between the levels of these biologically active compounds and beneficial health effects, it is neccesary to quantify accurately their levels in foods and biological fluids. In this study, HPLC-MS/MS method for the determination of isoflavones genistein, daidzein, and estrogenic metabolite-equol in bovine plasma and milk was optimised and validated. The method allowed low limits of detection: 5, 2.5 and 0.5 ng/ml for genistein, daidzein and equol, respectively, thus enabling to determine the effect of phytoestrogen-rich diet on the concentration of isoflavones and the metabolite in biological fluids of cows. The feeding experiment, carried out with four dairy cows, showed that a soy-based diet significantly increased both plasma and milk contents of biologically potent equol, therefore, the latter commodity could be an alternative source of this estrogenic metabolite, namely for the consumers who are not capable to convert it from the isoflavone precursors consumed in the diet.

Keywords: isoflavone; plasma; milk; LC-MS/MS

Isoflavones are secondary plant metabolites widely distributed in plant kingdom. The interest in these compounds started in the 80th of the last century, when epidemiological studies comparing Western and Asian diets indicated that a phytoestrogen-rich diet may prevent diseases such as breast and prostate cancer, cardiovascular diseases, osteoporosis, menopausal symptoms, etc. (Duncan *et al.* 2003; Cornwell *et al.* 2004). The putative beneficial health effects of isoflavones are based on their structural similarity to the mammalian estrogen 17β-estradiol. Due to this analogy, they may compete with estrogens in binding to estrogen receptors and show weak estrogenic activities (Kinjo *et al.* 2004).

The richest sources of isoflavones are soya beans, legumes, and clover. These plants often comprise substantial part of dairy cows feed, thus presenting a potential source of isoflavones (Figure 1) in bovine milk and milk products (Andersen *et al.* 2008). In addition, relatively high levels of equol could be found here. This potent estrogenic

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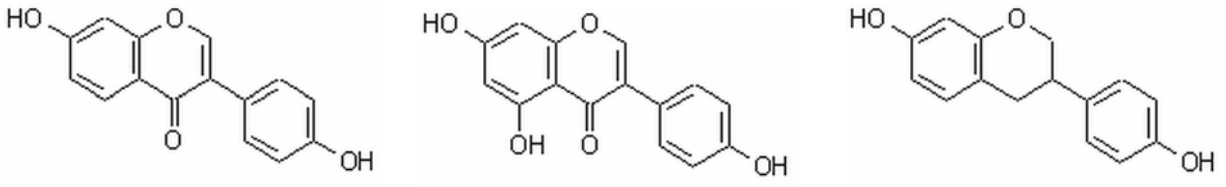

daidzein genistein equol

Figure 1. Structure of isoflavone aglycones and daidzein metabolite equol

metabolite of daidzein is produced by bacteria in the rumen of cows (Cederroth *et al.* 2009) and then, after absorption and transfer to plasma is excreted to milk. A similar process may occur in the metabolisation of daidzein in human intestine, nevertheless for non-equol producers, which represent one-third to one-half of the population (Yuan *et al.* 2007), bovine milk and its products could present a potential source of equol (Nielsen *et al.* 2009).

Only a few studies have been carried out to investigate isoflavone content in the products of animal origin such as bovine milk. The relationship between the way of farming and isoflavone content was investigated, rather higher levels of isoflavones were found in organically produced milk in comparison with conventionally managed dairy farms (Antignac *et al.* 2004; Hoikkala *et al.* 2007). Also in another studies, the effect of feedstuffs on the concentration and composition of phytoestrogens in milk was demonstrated (Andersen *et al.* 2008). The milk of cows fed red clover silage had significantly higher levels of isoflavones compared to that from cows fed white clover silage (Steinshamn *et al.* 2008). The concentrations of equol monitored in various cows milk samples ranged from 45 ng/ml to 582 ng/ml.

Various methods for the determination of phytoestrogens in biological samples have been reported. Plasma, urine, or milk isoflavone concentrations were determined employing high performance liquid chromatography (HPLC) coupled to coulometric array detection (CAD) (Nurmi *et al.* 1999) or by capillary electrochromatography (CE) (Starkey *et al.* 2002). A sensitive phytoestrogen analysis has been carried out using gas chromatography-mass spectrometry (GC-MS) (Grace *et al.* 2003; Heinonen *et al.* 2003). Currently the most often employed analytical technique in the analysis of phytoestrogens has become HPLC coupled with tandem mass spectrometry (MS/MS) (Morandi *et al.* 2005; Grace *et al.* 2007; Kuhnle *et al.* 2007; Rybak *et al.* 2008; Prasain *et al.* 2010) which provides high sensitivity, selectivity, and an easier sample preparation than GC-MS.

The aim of the present study was to evaluate the influence of soy-based feedstuff on the concentration of isoflavones in bovine plasma and milk. For this purpose, HPLC-MS/MS method was optimised and validated.

MATERIAL AND METHODS

Material. The samples of bovine blood plasma and milk were obtained from the Research Institute for Cattle Breeding, Ltd., Rapotín, Czech Republic.

The feeding experiment was carried out with four high-yielding lactating Holstein cows as described in detail by Třináctý *et al.* (2009). During this experiment, the cows were fed with soy-rich and control diets. Extruded full-fat soybeans, containing high levels of phytoestrogens (151 mg/kg of daidzein and 223 mg/kg of genistein) were used as soy-rich feedstuff. The control diet consisted of extruded rapeseed cake containing daidzein and genistein at concentrations below 3.5 mg/kg and 2.8 mg/kg, respectively. The feeding experiment was carried out as a replicated Latin square in a double reversal design and was divided into four periods of 42 days each (each period consisting of 21 days of preliminary phase and 21 days of experimental phase). Plasma and milk samples were taken three times a week, in total 9 samples were taken per each period.

The levels of soy isoflavones in the used diets were determined employing LC-DAD method. Briefly, 1 g of homogenised feed sample was hydrolysed with 10 ml of 6M HCl and 40 ml of 99% ethanol under the reverse condenser at the boiling point of ethanol for 4 hours. The extract solution was made up to 50 ml with 99% ethanol. Subsequently, the released aglycones were extracted from the

hydrolysate via solid-phase extraction (Oasis® HLB cartridge 3cc/60 mg, Waters, UK). The catridges were conditioned with 5 ml of methanol and 5 ml of water, after the loading of 8 ml of sample extract, the respective cartridge was washed with 5 ml of methanol:water (1:9, v/v). The target analytes were eluated by use of 6 ml of methanol. The sample extract was then evaporated to dryness and reconstituted in 2 ml of methanol:water (1:1, v/v). The analytes were separated on a reverse-phase column LiChrospher 100 RP18 (250 mm × 4.0 mm i.d., 5 µm, Merck KGaA, Darmstadt, Germany) by gradient elution of the mobile phase consisting of 1% acetic acid in water and methanol, the detection was carried out at 260 nm. The detection limits of daidzein and genistein were 3.5 mg/kg and 2.8 mg/kg, respectively.

Standards. Analytical standards of daidzein (≥ 98%), genistein (≥ 95%), equol (≥ 99%), and 4-hydroxybenzophenone (4-HBP) (≥ 99%) were purchased from Sigma-Aldrich (Steinheim, Germany). The individual stock standard solutions of daidzein, genistein, and 4-HBP (internal standard) were prepared in the concentration of 200 µg/ml in methanol, equol was dissolved in ethanol at the same concentration. The stock standard solutions were stored at –20°C. The working standard mixtures of 10 µg/ml were prepared by a serial dilution in methanol:water (1:1, v/v) and were subsequently used for the preparation of the solvent calibration standards which were stored at 4°C.

Chemicals. The enzyme β-glucuronidase/sulfatase (type H2, from Helix Pomatia, 11 400 U/ml of β-glucuronidase and 3290 U/ml of sulfatase) and acetic acid (glacial, 99.99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (for LC) and ethylacetate (for GC) were obtained from Merck KGaA (Darmstadt, Germany). Cyclohexane (PESTANAL®) was purchased from Riedel de Häen (Seelze, Germany). Deionised water was prepared using Milli-Q water system (Millipore, USA).

Sample preparation. *Blood plasma* – 0.5 ml of the plasma sample was incubated with 50 µl of enzyme β-glucuronidase/sulfatase (*Helix pomatia*) in 3 ml of sodium acetate buffer (0.1M, pH 5.0) at 37°C for 12 hours. After hydrolysis, the internal standard 4-HBP was added at the final concentration of 100 ng/ml. Subsequently, the hydrolysate was extracted by shaking with 5 ml of cyclohexane:ethylacetate (1:1, v/v). After centrifugation (11 180 × g, 5 min), a 3 ml aliquot was evaporated to dryness by rotary evaporation (40°C, 220 mbar) and redissolved in 0.5 ml of methanol: water (1:1, v/v). The final extract was analysed by LC-MS/MS.

Bovine milk – 2 ml of the milk sample were incubated with 100 µl of β-glucuronidase/sulfatase (*Helix pomatia*) in 3 ml of sodium acetate buffer (0.1M, pH 5.0) at 37°C for 12 hours. Then the internal standard 4-HBP was added at the final concentration of 100 ng/ml and the enzyme hydrolysate was extracted by shaking with 3 × 2 ml of ethylacetate. Three obtained organic phases were combined and evaporated to dryness. The dried extract was redissolved in 0.5 ml of methanol:water (1:1, v/v) and subsequently analysed by LC-MS/MS.

Chromatographic conditions. HPLC analyses were performed on HP1100 Binary Series LC system (Agilent Technologies, Santa Clara, USA). Chromatographic separation of the sample components was carried out on a reverse-phase column Discovery C18 (150 mm × 3.0 mm i.d., 5 µm, Supelco, Deisenhofen, Germany) at a flow rate of 0.7 ml/min held at 45°C. The mobile phase consisted of 0.1% acetic acid in water (A) and methanol (B). The gradient was as follows: 0–10 min, linear gradient from 50% to 75% B, 10.1–12 min, isocratic at 100% B, 12.1–16 min isocratic at 50% B to equilibrate. The total running time was 16 min for each sample. The sample injection volume was 10 µl.

The detection was performed with an ion trap mass analyser (LCQ Deca, Finnigan, USA) employing an atmospheric pressure chemical ionisation (APCI) source operating in the positive mode. The capillary and vaporiser temperatures were set at 200°C and 450°C, respectively. Discharge needle voltage was 6 kV. Nitrogen sheath gas flow and aux gas flow were 1.2 l/min and 3.0 l/min, respectively. For collision-induced dissociation (CID), helium was used as the collision gas. Scanning of ions was carried out in the selected reaction monitoring (SRM) mode. The parent and daughter ion combinations, retention times, and individual parameters for each compound used in SRM method are shown in Table 1.

Method performance characteristic. *Quantification* – The target analytes were quantified by means of calibration curves made on the basis of known concentrations in the mixtures of analyte standards with constant level of the internal standard 4-HBP (100 ng/ml). Nine calibration levels were used (2.5, 5, 10, 20, 50, 100, 200, 500, 1000 ng/ml). The calibration lines were obtained

Table 1. Monitored transitions and individual parameters used in LC-MS/MS detection of isoflavones

Compound	Quantification transition	Isolation width (*m*/*z*)	Activation amplitude (%)	Activation	Activation time (ms)	Retention time (min)
Daidzein	255.3 > 199.3	3	38	0.35	35	2.6
Genistein	271.4 > 215.3	2	37	0.35	35	3.1
Equol	243.1 > 123.1	2	29	0.30	40	3.4
4-HBP	199.2 > 121.2	3	30	0.35	35	4.0

by plotting the response factor (area analyte/area internal standard) against the concentration of the calibration standard.

Matrix effects – For the evaluation of potential ion supression or enhancement effects, the responses of the standard mixture solutions in the concentration range of 2.5–500 ng/ml (4-HBP at concentration 100 ng/ml) were compared with the responses of the matrix-matched solutions at the same levels. The matrix-matched standards were prepared using plasma and milk extracts containing low levels of the target analytes.

Limits of detection, limits of quantification – The limits of detection (LODs) and limits of quantification (LOQs) were defined as the concentrations at which the signal-to-noise ratio (S/N) was about 3 and 10, respectively.

Recovery – The recovery of the target analytes was determined by spiking the samples at two concentrations, 50 ng/ml and 100 ng/ml (milk) and 100 ng/ml and 500 ng/ml (plasma). The spiked samples were extracted following the described procedure.

Repeatability – The repeatability, expressed as a relative standard deviation (RSD), was determined by repetitive (n = 6) sample extractions and subsequent LC-MS/MS analyses.

RESULTS AND DISCUSSION

Optimisation of extraction and enzymatic hydrolysis

In the first experiment phase, the analytical procedure for two major soy phytoestrogens and the metabolite was implemented and validated. The optimisation of the individual steps is described below.

The major soy isoflavone compounds, genistein and daidzein, are linked (conjugated) to a sugar molecules to form glycosides. Most of the analytical strategies are designed to determine total isoflavone concentrations thus requiring a hydrolysis step to convert glycosides to the respective aglycones (Key *et al.* 2006). For the selected enzyme, β-glucuronidase/sulfatase (type H2, from Helix Pomatia, 11 400 U/ml of β-glucuronidase and 3290 U/ml of sulfatase) (Grace & Teale 2006), the amount required to hydrolyse all glycosides was determined. 50, 100, 200, 300 and 500 µl of the enzyme in 3 ml of sodium acetate buffer (0.1M, pH 5.0) were added to 0.5 ml of the plasma sample and to 2 ml of the milk sample and incubated at 37°C for 12 hours. The optimal yields of the target analytes were achieved at 50 µl and 100 µl enzyme additions to the plasma and milk samples, respectively. No substantial increase in aglycone yield was observed when using more than these amounts of β-glucuronidase/sulfatase.

To obtain acceptable recoveries of the target analytes from the plasma and milk samples, various medium polar extraction solvents were tested. When comparing the results obtained with methyl-*tert.*-buthyl ether, diethylether and ethylacetate, the last one was identified as the best option for the milk samples extraction. For the plasma samples, the mixture ethylacetate:cyclohexane (1:1, v/v) was used to provide a better phase separation and recovery as well. The final analytical scheme of the analysis of isoflavones in milk and plasma is shown in Figure 2.

Chromatographic and detection conditions

High performance liquid chromatography coupled to ion trap mass spectrometer was employed for the separation and detection of daidzein, genistein, and equol in bovine plasma and milk. When using Discovery C18 analytical column and gradient elution with mobile phase (consisting of methanol and 0.1% acetic acid in water), the separation of the target analytes was completed within 5 minutes. The

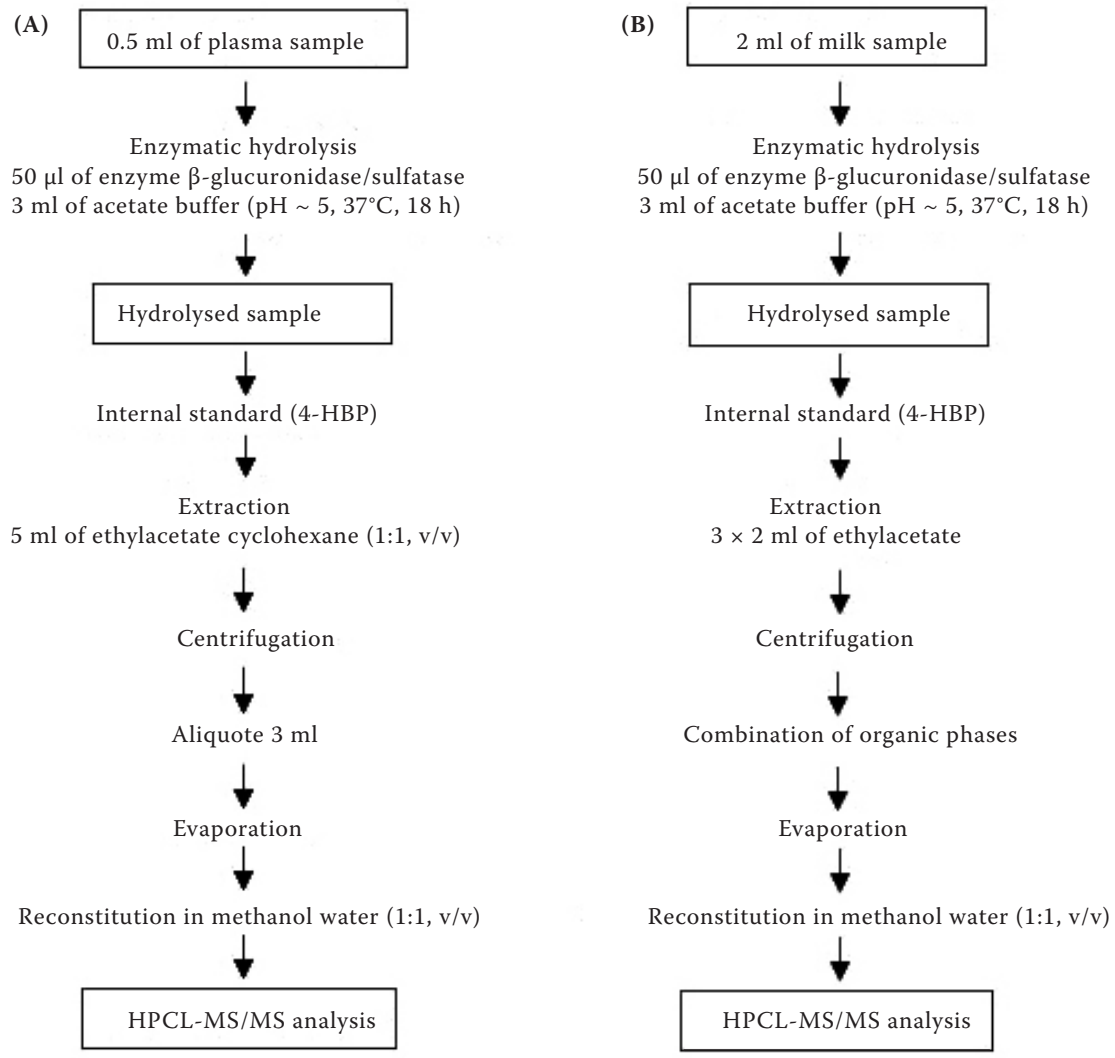

Figure 2. Analytical flow chart for analysis of isoflavones in plasma (A) and milk(B) samples

chromatogram of real plasma sample is shown in Figure 3. Both electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) were tested, the positive ions obtained by the latter one were the most intensive. The MS/MS transitions were optimised by infusions of pure individual standards at a concentration of 10 µg/ml in mobile phase methanol:0.1% acetic acid in water (1:1, v/v) at a flow rate 0.7 ml/ml. For quantification, the most abundant product ions were chosen and the second, less abundant ions were selected for confirmation.

Method validation

Quantification and matrix effects. The matrix components contained in the analysed samples may interfere with the ionisation process in MS source. Supposing they are co-eluted with the target analytes, typically ion suppression, or, occasionally, ion enhancement occurs (Avery 2003). If the standards in pure solvents are used for the calibration, the actual concentrations can be underestimated/overestimated. To assess these matrix effects, the calibration curves were constructed both for the solvent standard solutions and matrix-matched standards, prepared from plasma and milk with low contents of the target analytes. Comparing the calibration curve slopes, no significant matrix effects were found. On this account, the preparation of matrix-matched standards could be omitted, and quantification was carried out through external calibration curves obtained by plotting the standard concentrations (2.5–1000 ng/ml) versus the response ratios analytes/internal standard 4-HBP (100 ng/ml).

Table 2. Validation parameters of LC-MS/MS method for determination of isoflavones in bovine plasma and milk

Compound	LOD	LOQ	RSD (%)		Recovery (%)			
					plasma (ng/ml)		milk (ng/ml)	
	(ng/ml)		plasma	milk	100	500	50	100
Daidzein	2.5	8	5.0	4.8	87	104	102	104
Genistein	5	15	6.8	5.8	82	112	89	104
Equol	0.5	2	3.5	2.4	81	101	99	96

Performance characteristics were determined within an extensive validation study, their overview is given in Table 2. LODs and LOQs were determined by repetitive analysis (n = 5) of low levels solvent standard solutions and were defined as the concentration of each component for which the signal-to-noise ratio of 3 and 10, respectively, was obtained. The values of LOD and LOQ for daidzein, genistein, and equol were in the range of 0.5–5 ng/ml and 2–15 ng/ml, respectively. The recoveries of isoflavones in plasma and milk ranged from 81% to 112%, an increase with the spiking level was observed. The repeatabilities of the measurements, determined by analysing six times the milk and plasma extracts, ranged from 2.4% to 5.8 % and from 3.5% to 6.8%, respectively.

Phytoestrogens in biological samples

Soy, a common component of dairy cattle diet, is a rich source of energy due to its high contents of proteins and oil (Chouinard *et al.* 1997). In addition to these important nutrients, soy-based

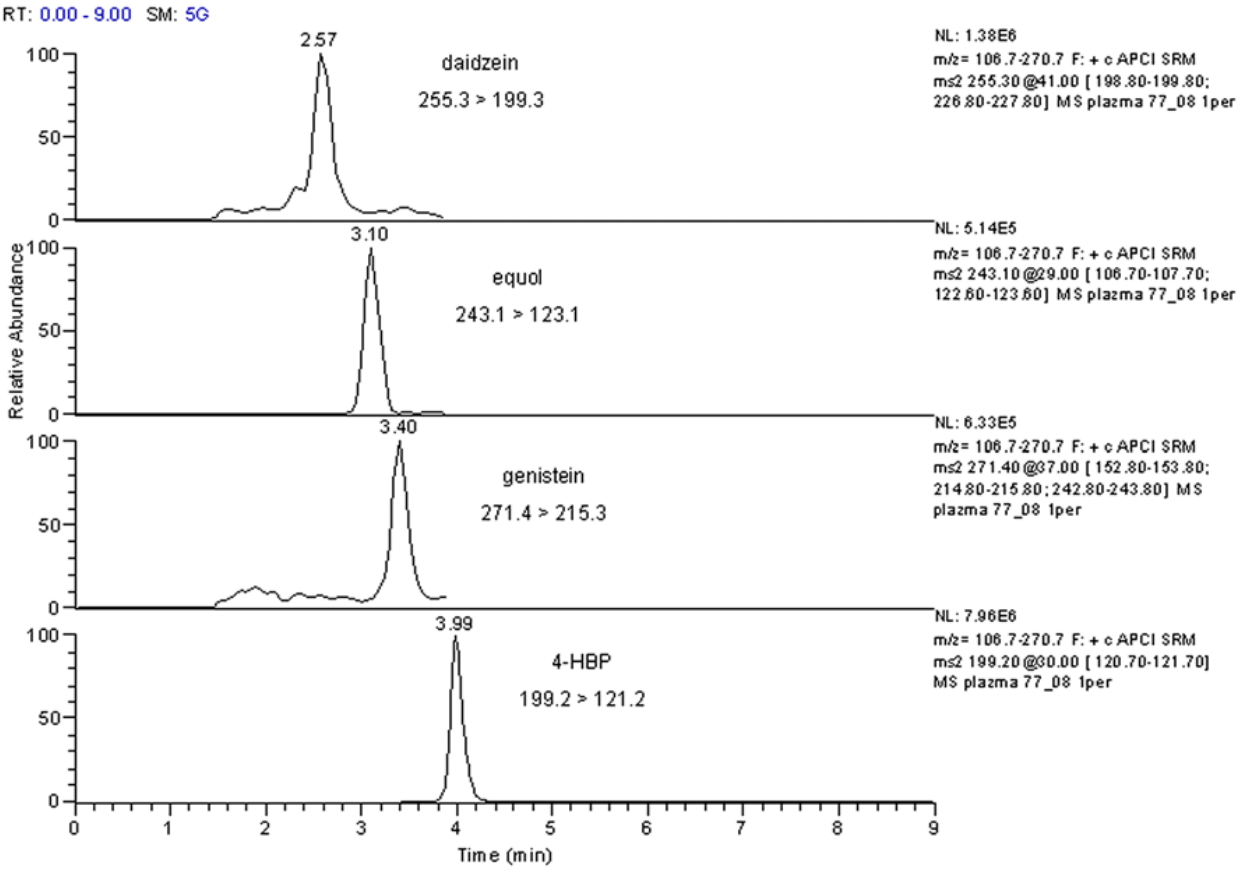

Figure 3. Chromatogram of plasma sample containing 38 ng/ml of daidzein, 196 ng/ml of equol, 69 ng/ml of genistein, and 100 ng/ml of 4-HBP

Table 3. Isoflavone concentrations in feeding experiments (result ± standard deviation)

	Dairy cow I		Dairy cow II		Dairy cow III		Dairy cow IV	
	control diet	soy-based diet	control diet	soy-based diet	control diet	soy-based diet	control diet	soy-based diet
Isoflavones intake (mg/day)								
Daidzein	29 ± 4	1496 ± 54	33 ± 1	1467 ± 52	31 ± 0.4	1143 ± 2	34 ± 1	1169 ± 16
Genistein	26 ± 1	2214 ± 48	27 ± 1	2219 ± 61	25 ± 0.4	1715 ± 37	28 ± 1	1764 ± 45
Concentration in plasma (ng/ml)								
Daidzein	14 ± 1	31 ± 6	14 ± 2	69 ± 9	16 ± 9	58 ± 32	10 ± 6	41 ± 10
Genistein	36 ± 13	96 ± 7	41 ± 33	58 ± 10	51 ± 5	95 ± 24	43 ± 36	66 ± 1
Equol	28 ± 20	226 ± 6	9 ± 4	203 ± 3	19 ± 10	220 ± 33	17 ± 8	227 ± 8
Concentration in milk (ng/ml)								
Daidzein	16 ± 0.2	17 ± 0.2	14 ± 0.5	19 ± 1.8	11 ± 0.6	13 ± 1.3	10 ± 0.2	14 ± 3.0
Genistein	57 ± 1.1	55 ± 5.4	51 ± 0.1	59 ± 0.1	16 ± 0.1	18 ± 4.4	16 ± 1.3	18 ± 4.4
Equol	4 ± 1.1	38 ± 14.4	3 ± 0.8	38 ± 4.0	4 ± 1.1	70 ± 1.4	3 ± 0.9	76 ± 38.0
Secretion into milk* (μg/day)								
Daidzein	379 ± 3	505 ± 32	312 ± 2	443 ± 23	226 ± 13	316 ± 28	225 ± 5	285 ± 56
Genistein	1382 ± 12	1676 ± 19	1146 ± 92	1399 ± 195	330 ± 3	425 ± 2	367 ± 48	372 ± 84
Equol	101 ± 27	1128 ± 341	63 ± 16	808 ± 136	81 ± 24	1690 ± 38	65 ± 26	1557 ± 760
Apparent recovery from feed to milk** (μg/mg)								
Daidzein + equol	16.6 ± 2.8	1.1 ± 0.2	11.6 ± 0.7	0.9 ± 0.04	10.0 ± 1.1	1.8 ± 0.1	8.2 ± 0.3	1.6 ± 0.7
Genistein	53.4 ± 0.6	0.8 ± 0.04	42.9 ± 4.5	0.6 ± 0.07	13.0 ± 0.1	0.2 ± 0.01	13.1 ± 1.3	0.2 ± 0.06

*calculated on the basis of measured isoflavones levels and milk yield per day; **recovery of daidzein (μg/mg) = (sum of daidzein and equol secreted into milk)/(sum of daidzein intake), recovery of genistein (μg/mg) = (sum of genistein secreted into milk)/(sum of genistein intake) (Steinshamn *et al.* 2008)

feedstuff is also a source of phytoestrogens which can be excreted into milk and thus transferred into the human food chain. The relationship was investigated between the administration of soy-based diet and the levels of phytoestrogens, daidzein, genistein, and metabolite equol in dairy cows plasma and milk. A general outcome of the feeding experiment was published earlier by Třináctý *et al.* (2009), the impact of the diet on sugars, proteins, fats, cholesterol and other metabolites in plasma and milk was investigated. In the current study, we bring more detailed information about the dynamics of isoflavones in biological fluids obtained from four experimental cows. The 168 days feeding experiment consisted of two cycles within which the animals were fed control (42 days) and soy rich (42 days) diets. The daily intake of isoflavones, their content in bovine plasma and milk, and the apparent recoveries from feed to milk are summarised in Table 3.

A higher dietary intake of isoflavones during soy-based periods resulted in a significant increase (*t*-test at 95% confidence level) in plasma levels of all target compounds in all experimental animals (compared to the control). The cows fed soy-based diet ingested daily on average 1319 mg and 1978 mg of daidzein and genistein, respectively. It should be noted that the control diet was not completely free of isoflavones, nevertheless the dietary exposure of animals was significantly lower (32 and 27 mg/day of daidzein and genistein, respectively). In Figure 4, the dynamics in plasma of three monitored isoflavones is shown (each bar represents the average result for four cows in one sampling). While the levels of equol increased by more than one order of magnitude immediately

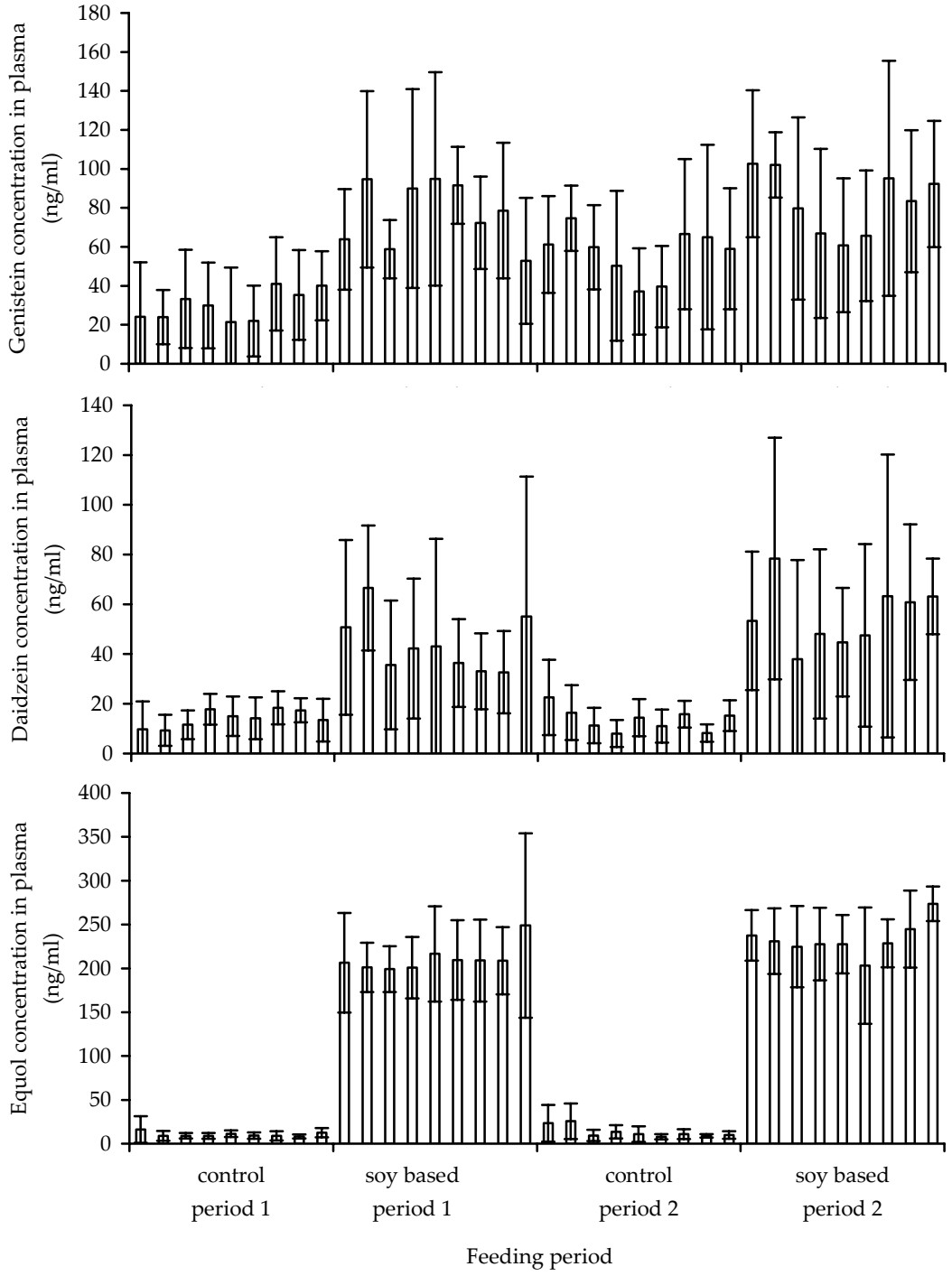

Figure 4. The fluctuation of plasma concentration of genistein, daidzein, and equol in dependence on isoflavone intake

after the start of the soy-rich diet period and rapidly dropped at its end, the differences between these periods in daidzein plasma concentrations were not so pronounced, although some increase of the target compound occurred as a response to the higher intake of this isoflavone from the diet. Even less distinct fluctuations were observed for genistein.

Regarding the levels of isoflavones in milk, significantly higher (*t*-test at 95% confidence level) equol amount was found again in milk from cows on the soy-based diet as compared to the control group. As illustrated in Figure 5 (each bar represents the average result for four cows in one sampling), the dynamics of equol in milk was almost identical

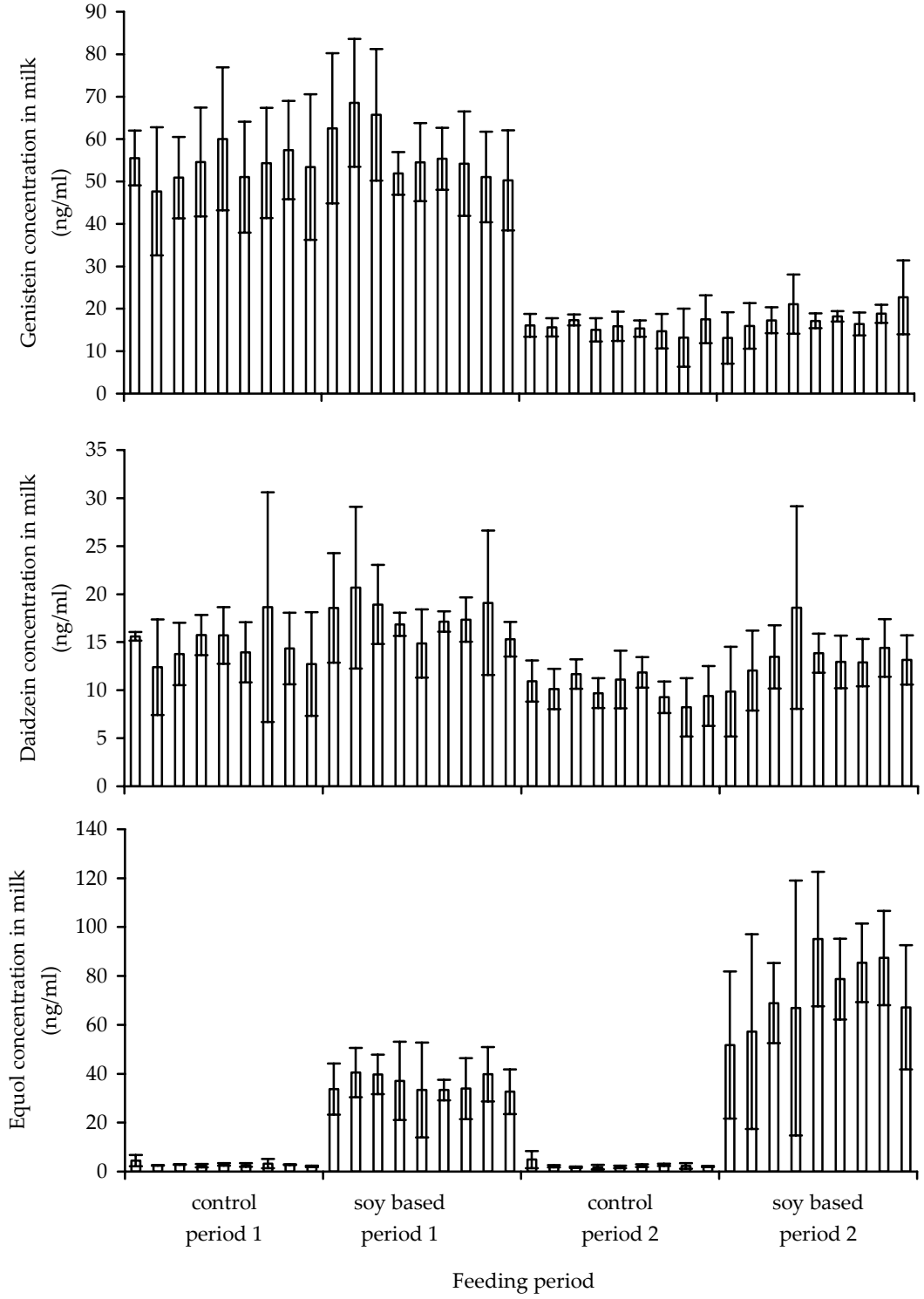

Figure 5. The influence of soy-based and control diet on the content of genistein, daidzein, and equol in milk samples

with that measured for plasma, nevertheless, its concentrations in this biological fluid were lower. Interestingly, the second soy rich period resulted, in spite of the identical dietary dose, in higher equol concentrations in milk. Rather an unexpected excresion profile was observed for genistein, high levels of this isoflavone were present even in the first control period with a following drop in the second one while subsequent administration of soy-rich feed did not increase its concentration.

The apparent recovery of isoflavones from feed to milk was expressed according to Steinshamn

et al. (2008) as the sum of daidzein and equol secreted into milk/sum of daidzein intake or as the sum of genistein secreted into milk/sum of genistein intake. The recovery grew with the decrease of isoflavone intake, which could be explained by the rate-limitation of the metabolism of phytoestrogens from feed in gastrointestinal tract and the hepatic reconjugation before blood transport and secretion into the udder (Steinshamn *et al.* 2008). More studies are needed to clarify this metabolic process.

CONCLUSIONS

In this study, we documented the transfer of phytoestrogenic isoflavones from feed to cow milk.

The outcome of the experiments can be summarised as follows:

– A fast and simple HPLC-MS/MS method was developed and validated for the determination of isoflavone aglycones, daidzein, genistein, and metabolite equol in bovine plasma and milk.
– Following the administration of feedstuff rich in daidzein, a rapid increase of equol, both in plasma and milk, occurred. No accumulation of this compound in dairy cows biological fluids was observed.
– An increased intake of daidzein and genistein by the cows resulted in the elevated levels in plasma, however, in milk only a weak dependence on the experimental diet (soy-based vs. control) was observed for daidzein. For genistein, no increase was found in the second part of the feeding experiment in response to this isoflavone intake.
– Futher investigations in this field are needed to describe the ruminants metabolism of phytoestrogens and potential beneficial effects of these compounds on human health.

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