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New data regarding phytoestrogens content in bovine milk

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Abstract

This study investigates the occurrence of the main phytoestrogens in various commercial bovine milk samples, representing the main foodstuff of a critical population. The results show the presence of isoflavones at trace levels (i.e. in the 0.1–5.0 $\mu g l^{-1}$ range) in most of the samples, and the presence of equol and enterolactone in all analyzed samples at relatively high concentrations (14.1–293 and 14.3–94.4 $\mu g l^{-1}$, respectively). The consequent potential estrogenic effect on specific populations of children could be questioned. New data are also provided regarding first the correlation between the concentrations of the different isoflavones and second the influence of the animal feedstuff on the milk phytoestrogen content.

Keywords: Endocrine disruptors; Phytoestrogens; Isoflavones; Lignans; Equol; Enterolactone; Milk; Exposure assessment; Mass spectrometry; LC-MS/MS

1. Introduction

In an international context, scientific research on food safety, especially regarding chemical risk assessment through the food chain and endocrine disruptor chemicals (EDCs) now receives increased attention. Among the large and increasing range of compounds recognized, or suspected, to be EDCs, naturally occurring substances, such as phytoestrogens remain poorly investigated compared to molecules of industrial origin, such as dioxins, PCBs, bisphenol A or alkylphenols. Numerous papers have described to the potential positive effects of phytoestrogens, for instance on menopause symptoms (Anderson & Garner, 1997; Setchell & Cassidy, 1999) or cardiovascular diseases (Mishra et al., 2000; Tikkanen & Adlercreutz, 2000; Wroblewski Lissin & Cooke, 2000). Others have highlighted their estrogenic properties and possible adverse-effects on health, especially in case of exposure at critical stages of development (Adlercreutz, 1999; Bennetau-Pelissero, 2001; Diels et al., 2000; Jefferson & Newbold, 2000; Lephart et al., 2002; Setchell & Cassidy, 1999; Whitten & Patisaul, 2001).

Their occurrence has been mainly evaluated in vegetables (Clarke et al., 2003; Liggins, Bluck, Runswick, Atkinson, Coward, & Bingham, 2000a, 2000b; Mazur & Adlercreutz, 2000; Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998; Nurmi, Heinonen, Mazur, Deyama, Sansei, & Adlercreutz, 2003). Their metabolism in rodents was also investigated (Coldham et al., 1999; King, Broadbent, & Head, 1996; Sfakianos, Coward, Kirk, & Barnes, 1997). Some methods were proposed for their measurement in human urine (Uehara et al., 2000; Valentin-Blasini, Blount, Schurz Rogers, & Needham, 2000), plasma (Adlercreutz et al., 1993; Lapèík, Hampl, Hill, Wähälä, Al-Maharik, & Adlercreutz, 1998) or in soy or breast milk (Bennetau-Pelissero et al., 2003; Dwyer, Goldin, Saul, Gualtieri, Barakat, & Adlercreutz, 1994; Franke, Yu, Maskarinec, Fanti, Zheng, & Custer, 1999; Slavin, 1997). Nevertheless, the occurrence of these molecules in food remains largely under investigated, especially in food products of animal origin as well as the exposure assessment of critical populations such as children. Therefore, the estimation of phytoestrogens in bovine milk appeared to be of great interest, especially because of increasing use of vegetal flours in

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animal feedstuffs, which may contain high amounts of isoflavones.

The purpose of the present study was to investigate the occurrence of the main phytoestrogens (isoflavones, lignans, coumestans) in various commercial bovine milk samples, using a previously described extraction/purification process coupled to an improved identification method based on liquid chromatography-electrospray tandem mass spectrometry (Antignac, Cariou, Le Bizec, Cravédi, & André, 2003). Target phytoestrogens were then measured in 26 different commercial milk samples, taking into account the influence of milk fat content (full cream or skim) as well as bovine alimentation type (classical or organic agriculture).

2. Materials and methods

2.1. Reagents and chemicals

Sodium acetate was purchased from Merck (Darmstadt, Germany). Pestipur or HPLC grade solvents (acetone, methanol, cyclohexane, acetic acid, ethyl acetate) were from Solvents Documentation Synthesis (SDS, Peypin, France). Enzymatic preparation was a purified lyophilized extract from *Helix pomatia* (Sigma, St Louis, MO, USA) dissolved in water (25000 sigma units/ml). Reference phytoestrogens (Fig. 1) were provided by Sigma-Aldrich. Standard solutions were prepared at 1 mg ml^{-1} in methanol/DMSO (95:5, v/v). Working solutions were prepared by successive tenfold dilutions, at concentrations from 100 to 1 ng μl^{-1}. All these solutions were stored in the dark at −18 °C.

2.2. Sample preparation

To the milk sample (10 ml) were added 50 ng of the internal standard (prunetine), 2 ml acetate buffer (2M, pH 5.2) and 8 ml acetone. After stirring (vortex, 1 min) and centrifugation (15 min, 4500 rpm), supernatant was removed and evaporated under a nitrogen stream at 45 °C to a 2-fold reduced volume. Thus, 400 µl of the enzymatic preparation were added, and the reaction was performed at 52 °C during 4 h, allowing hydrolysis of the conjugated phase II metabolites (glucuronide and sulfate forms). After centrifugation (15 min, 4500 rmp), the supernatant was applied onto a C_{18} SPE cartridge (1 g solid phase, SDS, Peypin, France) previously activated with 10 ml methanol and 10 ml water. After a washing step with 10 ml water, analytes were eluted with 6 ml methanol. The extract was evaporated to dryness under a nitrogen stream at 45 °C and reconstituted in 0.1 ml ethyl acetate + 0.4 ml cyclohexane. The extract was then applied onto a SiOH SPE cartridge (0.5 g solid phase,

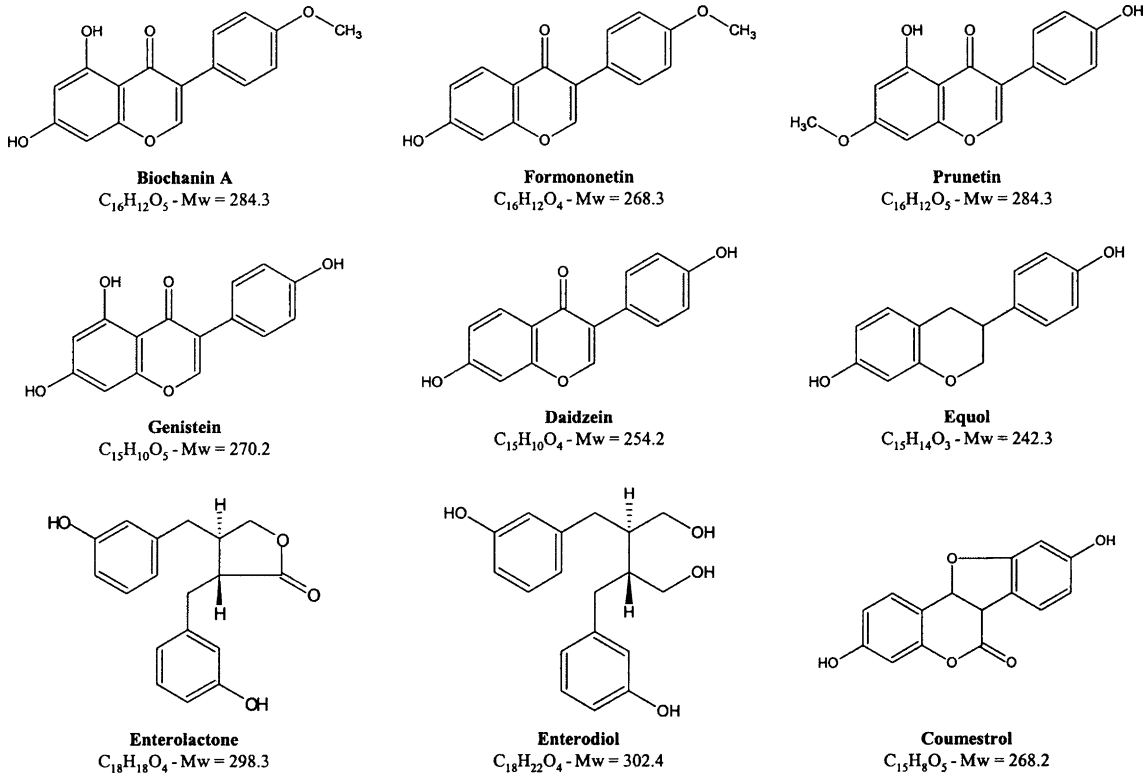

Fig. 1. Chemical structures of the investigated phytoestrogens.

SDS, Peypin, France) previously activated with 20 ml cyclohexane. After a washing step with 10 ml cyclohexane/ethyl acetate (90:10, v/v), analytes were eluted with 10 ml cyclohexane/ethyl acetate (20:80, v/v). The extract was evaporated to dryness under a nitrogen stream at 45 °C and reconstituted in 50 μl methanol/water/acetic acid (30:70:0.5, v/v/v).

2.3. HPLC separation

An Alliance® 2690 HPLC pump with quaternary gradient system and automatic injector was used (Waters, Milford, MA, USA). Reversed phase liquid chromatography separation was achieved on octadecyl grafted silica stationary phase Nucleosil® C_{18}AB (50×2.1 mm, 5 μm + guard column 10×2.1 mm) from Macherey-Nagel (Düren, Germany). Elution solvents were methanol (A) and water containing 0.5% (v/v) acetic acid (B). Mobile phase composition (A:B, v/v) was 30:70 at 0 min, 100:0 from 13 to 15 min, and 30:70 from 20 to 30 min. Flow rate was 0.3 ml/min and injected volume was 10 μl.

2.4. LC-MS/MS identification

Mass spectrometric data were acquired using a QuattroLC® (Waters-Micromass, Manchester, UK) triple quadrupole operating in negative electrospray (ESI-) ionization mode and multiple reaction monitoring (MRM) acquisition mode. Nitrogen was used as nebulization and desolvation gas, at flow rates of 90 and 600 l/h, respectively. Source and desolvation temperatures were set at 130 and 400 °C, respectively. Potentials applied to the capillary (from 2.5 to 4.0 kV) and to the cone (from 15 to 35 V) were evaluated and optimized for each analyte. Argon was used as collision gas at a pressure of 4.0×10^{-4} mBar, collision energy varying from 2 to 30 eV. Two diagnostic MRM transitions were recorded for each analyte.

2.5. Data analysis

Calibration curves were included in each analysis batch, using 3–5 spiked and extracted water samples at concentrations varying from 1 to 25 $\mu g\,l^{-1}$. For each analyte, quantification was based on the more intense MRM diagnostic transition, considering the analyte/internal standard relative signal amplitude. Statistical analyses were performed with Statistica® software (v 5.5 for Windows, Statsoft, Maison-Alfort, France). These analyses included classical descriptive statistics (average, relative standard deviation, correlations) as well as Student *t*-test and linear discriminant analysis (LDA) in order to check for eventual differences among the analyzed milk samples regarding their phytoestrogen contents.

3. Results

Each analysis batch was validated considering the following classical procedure: presence of all the target analytes in the extracted spiked control samples, no cross contamination in the extracted blank control samples, and presence of the internal and external standards in all of the samples. Each calibration curve used for quantification was characterized by a coefficient of determination (R^2) better than 0.97. The unambiguous identification of the analytes found in the analyzed samples was based on the EC/2002/657 decision criteria. This consisted of verifying the retention time, the signal to noise ratio of at least two diagnostic MRM transitions monitored in LC-MS/MS, and the ratio of intensities between these different diagnostic signals, in accordance with specified tolerances (Antignac et al., 2003). Limits of identification were estimated on the basis of the observation of the ion chromatograms obtained for the low concentration calibration point in terms of signal to noise ratio and subsequent proportionality rule.

The estimated concentrations determined for the target phytoestrogens in the tested milk sample are given in Table 1, while Fig. 2 presents the diagnostic ion chromatograms obtained for one specific milk sample. The first observation was the absence of enterodiol and coumestrol in all analyzed milk samples, with a detection limit proved to be 0.5 ppb. The second result was the presence of isoflavones at trace levels (i.e. in the 0.1–5.0 $\mu g\,l^{-1}$ range) in most of the samples, in both their methoxylated (Formononetin, Biochanin A) and hydroxylated (Daidzein, Genistein) forms. The last and probably more significant result was the presence of equol and enterolactone in all analyzed samples at relatively high concentrations (14.1–293 and 14.3–94.4 $\mu g\,l^{-1}$, respectively).

The Student *t*-test authorized the comparison of the classical/biological organic milk samples and skimmed/full creamed milk samples in terms of phytoestrogen content. Results are given in Table 3, which revealed

Table 1
Estimated concentrations ($\mu g\,l^{-1}$) determined for each measured phytoestrogen in each analyzed milk sample

	μ	sd	min	max	LOI
Enterodiol	nd	nd	nd	nd	0.1
Coumestrol	nd	nd	nd	nd	0.5
Formononetin	1.2	1.5	0.1	5.0	0.1
Biochanin A	0.7	0.9	0.0	2.8	0.1
Daidzein	1.8	2.3	0.0	9.6	0.5
Genistein	0.9	1.7	0.0	5.8	0.5
Equol	78.0	79.3	14.1	293.0	0.5
Enterolactone	40.0	19.9	14.3	94.4	0.1

nd: non detected; μ: average; sd: standard deviation; min: minimum observed value; max: maximum observed value; LOI: estimated limit of identification.

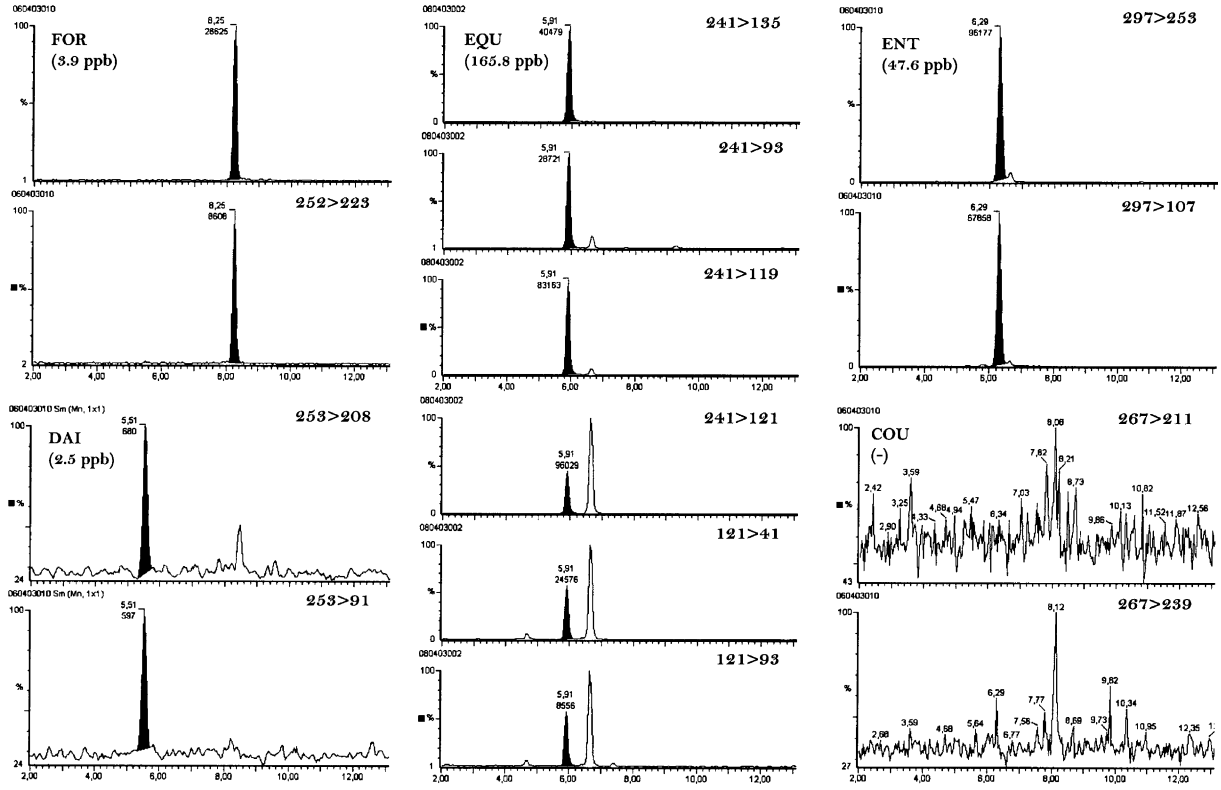

Fig. 2. Diagnostic ion chromatograms of 5 phytoestrogens in a specific milk sample from organic agriculture (FOR: formononetin; DAID: Daidzein; EQU: Equol; ENL: Enterolactone; COU: Coumestrol; –: non-identified; x ppb: estimated concentration in $\mu g/l^{-1}$).

significant differences between classical and organic agriculture milk samples regarding the concentration of all isoflavones (99% confidence level for formononetine, biochanine A, daidzein, equol and 95% confidence level for genistein). Fig. 3 shows the representation of the total 26 analyzed milk samples on the two main axes extracted by a linear discriminant analysis, which confirmed the possible discrimination between milk samples. The corresponding analysis parameters are given in Table 4.

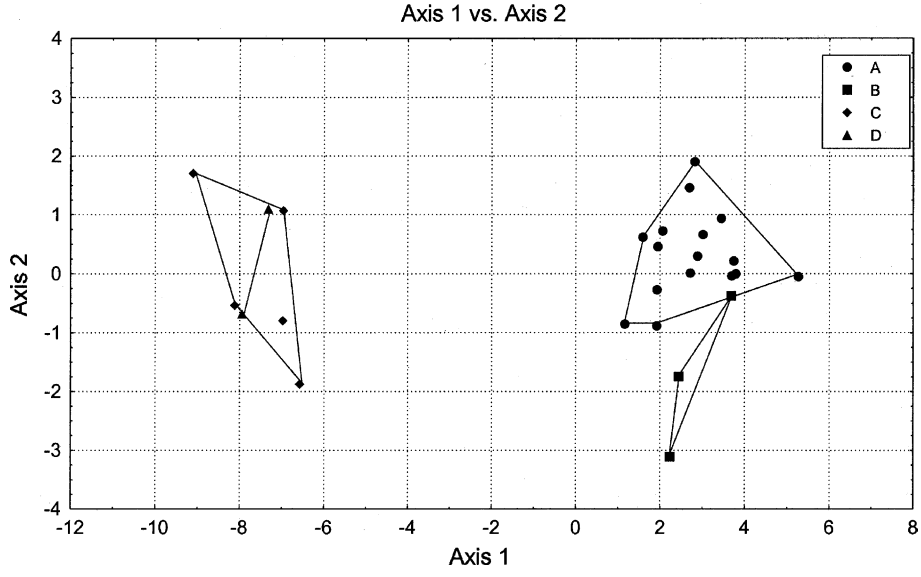

Fig. 3. Representation of the total 26 analyzed milk samples on the two main axes extracted by a linear discriminant analysis (A: classical agriculture and skimmed samples, B: classical agriculture and full cream samples; C: organic agriculture and skimmed samples; D: organic agriculture and full cream samples).

4. Discussion

4.1. Concentration of phytoestrogens in milk samples

The observed values remain between 10 and 100 times lower than the concentrations generally reported for isoflavones in specific food products such as soy-based milk formulae or tofu. However, the question of their estrogenic potency and potential adverse effect on critical populations such as children should be posed. As shown in Table 2, unsurprising significant coefficients of correlation were observed between the concentrations of the different isoflavones in general and between equol and its metabolic precursors in particular. However, the observed correlation coefficient between equol and its methoxylated precursor formononetin appeared significantly higher than the one with its hydroxylated precursor daidzein (0.95 vs 0.69, respectively). If confirmed on a large number of additional samples, this result should be investigated in terms of metabolic pathways of isoflavones in cattle.

4.2. Discrimination among milk samples

The differences observed between milk samples from classical and organic agriculture might be explained by the type of feedstuff used for animal breeding. Indeed, organic agriculture practices are supposed to use vegetal products with enriched nutritional properties such as soy or colza flours rather than hay- or maize-derived products. So these results seems to demonstrate the influence of the animal feeding on the milk phytoestrogen content. Other factors, such as animal geographical origin, species, or gender have to be investigated, particularly to evaluate exposure assessment of these molecules. The second observation in Table 3 is the non-significant difference between skimmed and full cream milk samples regarding their phytoestrogen contents. This observation is not very surprising, phytoestrogens, being not particularly lipophilic compounds; moreover, some of our results demonstrate a large majority of conjugated forms in milk (data not shown). The LDA analysis (Fig. 3) confirmed a possible discrimination

Table 2
Correlation coefficients between the statistical variables associated with the concentrations of the different plytoestrogens

Variable	Formononetin	Biochanin A	Daidzein	Genistein	Equol	Enterolactone
Formononetin	1.00	–	–	–	–	–
Biochanin A	0.75**	1.00	–	–	–	–
Daidzein	0.62**	0.70**	1.00	–	–	–
Genistein	0.31	0.57**	0.73**	1.00	–	–
Equol	0.95**	0.74**	0.69**	0.35	1.00	–
Enterolactone	0.14	0.40*	0.61**	0.34	0.20	1.00

*/**: 95%/99% confidence level.

Table 3
Student *t*-test results regarding the influence of the milk sample type (classical vs organic agriculture) and fat level (full cream vs skimmed) on the phytoestrogen content

Analyte	Classical ($n = 19$)		Organic agriculture ($n = 7$)		t	p
	μ	sd	μ	sd		
A: Comparison between classical and organic agriculture samples regarding their phytoestrogen content ($\mu g/l^{-1}$)						
Formononetin	0.3	0.1	3.4	1.0	−13.9816	0.000000*
Biochanin A	0.3	0.6	1.8	0.4	−5.5737	0.000010*
Daidzein	1.0	1.4	3.9	3.0	−3.4200	0.002245*
Genistein	0.5	1.0	2.1	2.5	−2.4492	0.021997
Equol	36.4	14.8	191.0	72.0	−9.1528	0.000000*
Enterolactone	39.1	20.2	42.1	20.6	−0.3340	0.741273
	Full creamed ($n = 21$)		Skimmed ($n = 4$)			
	μ	sd	μ	sd		
B: Comparison between full cream and skimmed milk samples regarding their phytoestrogen content ($\mu g/l^{-1}$)						
Formononetin	0.9	1.3	1.1	1.4	0.19605	0.846293
Biochanin A	0.8	1.0	0.6	0.9	−0.33515	0.740549
Daidzein	1.2	1.7	1.5	1.8	0.34453	0.733578
Genistein	0.9	1.8	0.8	1.5	−0.16923	0.867097
Equol	72.0	70.5	68.9	68.7	−0.08130	0.935906
Enterolactone	47.3	17.1	36.4	18.1	−1.11425	0.276679

μ: average; sd: standard deviation; t: Student t-value; p: signification degree; *: significant difference at the 99% confidence level.

Table 4
Parameters of the linear discriminant analysis performed on the 26 analyzed milk samples (CVP: cumulated variance proportion)

Analyte	Axis 1		Axis 2	
	Coefficient	Correlation	Coefficient	Correlation
Formononetin	−3.58066	−0.571919	−0.288092	0.033356
Biochanin A	0.17408	−0.230497	0.645187	0.006765
Daidzein	0.95629	−0.142341	1.016998	0.138807
Genistein	−1.28075	−0.101022	−0.456082	0.163831
Equol	−0.00171	−0.379605	−0.013191	−0.035171
Enterolactone	−0.01327	−0.014324	−0.087818	−0.464889
Constant	4.21152	–	3.040064	–
CVP (%)	97.494		99.4346	

between milk samples from classical and organic agriculture on the basis of their phytoestrogen content profiles. More precisely, this discrimination appeared on the axis 1 and is mainly based on the concentrations of formononetin and equol (Table 4: high correlation coefficients between these variables and the axis 1). Even if a tendency is perceptible regarding the discrimination of full cream and skimmed milk samples on the basis of their contents of enterolactone (Table 4: slight correlation between this variable and axis 2), this observation has to be confirmed and extended to a large number of samples.

5. Conclusions

This study is, to our knowledge, the first exposure assessment of phytoestrogens in commercial bovine milk. An efficient analytical development for the purification and the unambiguous identification of the main phytoestrogens by LC-MS/MS allowed us to demonstrate the presence of isoflavones at trace concentrations and the presence of non-negligible amounts of equol and enterolactone in all the studied samples. Even if the observed concentrations remain lower than those reported in vegetable- or soy-enriched products, the estrogenic potential effect on specific populations could be questioned. In particular, this work demonstrates a direct way of exposure to equol, with potential consequences for children and people that are not able to realize the biotransformation of daidzein in equol (35% and 60% of the European and Asian populations, respectively). The relationships observed between the concentrations of the different analytes, as well as the discrimination observed between milk samples, depending of the animal feedstuff, are worthy of investigation in terms of phytoestrogen metabolism in cattle. For this purpose, the study of the biotransformations and the target tissues of phytoestrogens in cattle should be relevant. Finally, the relatively high measured concentrations of recognized estrogenic species, such as equol and enterolactone, merits more attention in terms of metabolism and toxicology in the human organism. According to several authors who have reviewed phytoestrogens and food, the need for additional and exhaustive analytical data is evident. The present study aimed to provide data on phytoestrogens in milk as a principal food for critical populations such as children. This should be extended in the near future to a large number of samples in other biological matrices of animal origin representing a potential risk to consumers.

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