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Research Article

High levels of equol in organic skimmed Finnish cow milk

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The isoflavonoids, equol, formononetin, daidzein, genistein, biochanin A, and *O*-demethylangolensin (*O*-DMA), were analyzed from commercial cartons of skimmed Finnish milk by HPLC-diode array detector (DAD)-FL. We found 411 ± 65 ng/mL of equol and traces of formononetin and daidzein in organic skimmed milk whereas conventionally produced milk contained 62 ± 16 ng/mL of equol and no formononetin or daidzein.

Keywords: Bovine milk / Equol / HPLC analysis / Phytoestrogens

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1 Introduction

Phytoestrogens have been under growing interest due to their suggested beneficial effects on humans. Soy is the most common plant estrogen source in human nutrition, containing the phytoestrogenic isoflavones, daidzein, and genistein. Also red clover incorporates isoflavones abundantly, mostly formononetin and biochanin A. These plant polyphenols have been shown to have both estrogenic and antiestrogenic properties. According to epidemiological studies, phytoestrogen rich diet, as in Southeast Asian countries, appears to diminish chronic diseases such as breast and prostate cancers, cardiovascular disease, and osteoporosis [1–3]. Also the possible adverse effects of these hormonally active agents are presently studied concerning food safety aspects in particular [4]. Their action as endocrine disruptor chemicals (EDCs) has been studied to some extent especially in regard to estrogenicity in cells of different estrogen sensitive tissues [5], as well as their androgen receptor binding activity [6].

Phytoestrogens, together with their mammalian metabolites have been isolated from various biological fluids. One of the most interesting metabolites of red clover or soy isoflavones in mammals is equol. Recent research has, for instance, shown equol to act as an antiandrogen that inhibits prostate growth and hormone feedback in rats [7]. Several *in vitro* or animal studies have indicated equol to possess a higher biological activity than the precursor compounds daidzein and formononetin [8–10].

Studies suggest that less than half of the human population are so-called equol producers [11–16]. This means that the gut microflora of such people is able to convert isoflavone precursors into equol. In contrast, all chimpanzees, rats and mice are equol producers [17] as well as sheep and cattle [18, 19]. We have very recently demonstrated that equol exists solely as the (*S*)-enantiomer in red clover fed ewes' serum [19]. There are very few studies, however, where the amounts of equol in food products have been analyzed. Bannwart *et al.* [20] identified equol for the first time in cow's milk in 1985 by GC/MS. In 1998, King *et al.* [21] studied the concentrations of daidzein, genistein, and equol in Australian bovine milk. Concentrations were assessed in milk from different areas, at different times of the year and after different dairy processing methods. Antignac *et al.* [22, 23] later reported a method for the quantitation of phytoestrogens in bovine milk by HPLC-ESI-MS/MS.

In cattle nutrition, forage plants such as grasses and legumes have an essential role. Especially in organic farming, where the use of fertilizers is limited, forage legumes are important due to their capability to bind nitrogen from air. Leguminous plants, clovers, and alfalfa in particular, contain significant amounts of phytoestrogens, isoflavones, and coumestans [24]. In Finland, red clover is the predominant legume cultivated in organic dairy farms. In conven-

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Abbreviations: DAD, diode array detector; ***O*-DMA,** *O*-demethylangolensin

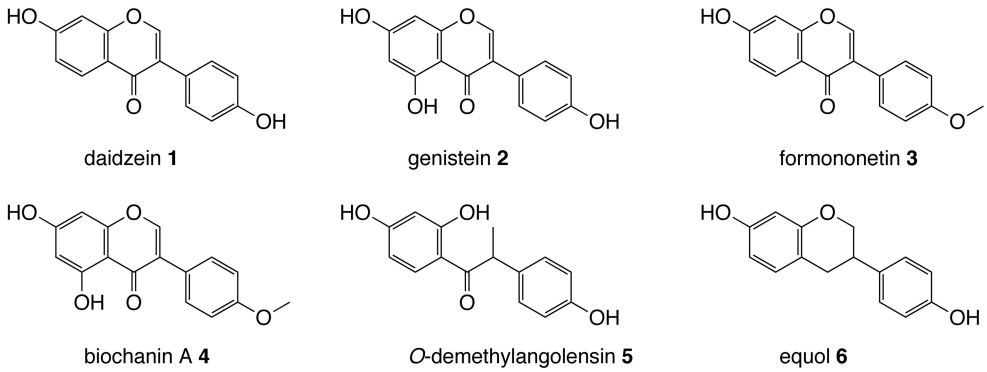

Figure 1. Phytoestrogens analyzed in the study.

tional nonorganic dairy production, however, the use of legumes is scarce, and grass silage based ratios supplemented with barley, oats, and rapeseed meal are the most typical dairy cow ratios during indoor feeding [25]. We have now assessed the amounts of phytoestrogens, daidzein, genistein, formononetin, biochanin A, *O*-demethylangolensin (*O*-DMA), and especially equol 6, in both organic and conventionally produced milk. Skimmed milk was chosen because its interest among consumers is steadily growing. Currently, it is the second most popular type of cow milk consumed in Finland.

2 Materials and methods

2.1 Materials

β-Glucuronidase (G-0751) and sulfatase (S-9626) were purchased from Sigma (St. Louis, MO, USA). Buffer solutions and diethyl ether were from Merck (Darmstadt, Germany) and were of p.a. grade. Methanol was from J. T. Baker (Deventer, The Netherlands) and ACN form Rathburn Chemicals (Walkerburn, UK). Both were HPLC-grade solvents. Water was purified using Millipore Milli-Q system (Billerica, MA, USA). Oasis HLB 3 cc (60 mg) SPE cartridges were purchased from Waters (Milford, MA, USA). Acrodisc LC 13 0.45 µm filters were purchased from Pall (New York, NY, USA).

Biochanin A was purchased from Aldrich (St. Louis, MO, USA), coumestrol and formononetin from Fluka (Buchs, Switzerland), and daidzein, genistein, and flavone from Sigma. Equol and *O*-DMA were synthesized in the Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, Finland.

2.2 Milk treatment

Twelve organic and four conventionally produced cartons of milk were purchased from grocery shops between January and March 2005. Milk was processed by the biggest dairy company in Finland (Valio), which collects milk all over the country from small dairy farms.

Flavone (200 µL, 12.5 µg/mL in ethanol) was added into a 50 mL vial for recovery assessment [26]. The solvent was evaporated under nitrogen and 5 mL of fat free milk was added together with 100 µL of β-glucuronidase (500 units) and 80 µL of sulfatase (40 units). The sample was incubated at 37°C for 2 h. Next, 1 mL of 1.5 M ammonium acetate buffer and 7.5 mL of diethyl ether were added and samples were vortexed for 3 min. Samples were then centrifuged at $4752 \times g$, chilled at −80°C, and recentrifuged at +4°C. The ether extract was removed and evaporated under a stream of nitrogen at +40°C. This procedure was repeated three times. Sodium acetate buffer (2 mL, 0.2 M, pH 5.0) containing 20% methanol was added and samples were centrifuged at $4752 \times g$ for 5 min at +4°C. The supernatant was then applied onto SPE cartridges that had been activated with 2 mL of methanol and 2 mL of water. After rinsing the cartridges with 5 mL of 5% methanol, samples were eluted with 10 mL of ACN/MeOH (9:1). Samples were evaporated under nitrogen at +60°C and reconstituted in 150 µL of methanol and 50 µL of 0.2 M sodium acetate buffer (pH 5.0). Samples were then centrifuged and the supernatant was filtered into a vial.

2.3 Instrumentation and conditions

Duplicate samples of milk were analyzed by HPLC by a method modified from Franke *et al*. [27], Lund *et al*. [7], and Antignac *et al.* [22]. Detection was by both UV-diode array detector (DAD) at 262 nm for flavone, genistein, *O*-DMA, and biochanin A and fluorescence detector (FLD) excitation 254 nm, emission 465 nm for daidzein and formononetin and emission 310 nm for equol.

The following analytical equipment was used: HPLC Column Zorbax Eclipse XDB-C18 4.6 mm × 250 mm part 7995118-585 Agilent Technologies; precolumn Zorbax R-P 4 mm × 4 mm, 7995118-504 Agilent Technologies. Liquid Chromatograph 1100 Degasser, Japan; Binary Pump, FLD,

Table 1. LODs and LOQs for the compounds analyzed

	LOD	LOQ
Daidzein 465 em	2.1	4.0
Genistein 262 nm	6.8	24.0
Equol 310 em	30.0	41.0
O-DMA 262 nm	26.9	Not determined
Formononetin 465 em	1.3	3.3
Biochanin-A 262 nm	7.3	22.5

Values are in ng/mL.

DAD, Agilent Technologies, Germany, ChemStation data system, Germany. Analytical conditions: linear gradient between 46% MeOH/10 mM $Na_2HPO_4 \cdot 2H_2O$ pH 6.5 and 100% methanol for 23 min, post-time 8 min, flow 1 mL/min, injection volume 10 µL. Column oven temperature was +40°C.

2.4 Identification

Isoflavones and metabolites were identified from duplicate milk samples using authentic reference compounds as external standards. Calibration curves were established in the concentration range of 1.172–37.5 µg/mL for equol and 0.391–12.5 µg/mL for the other compounds with correlation over 0.999. LODs and LOQs were determined at S/N over 3 and 10, respectively (Table 1). Identification was confirmed by comparing UV-spectra of the eluting peaks with reference compounds.

Flavone was used as a standard to calculate recovery. The recovery level for flavone was 80% ± 6%.

3 Results and discussion

Daidzein, genistein, formononetin, biochanin A, *O*-DMA, and equol contents were analyzed from 12 organic commercial milk samples (Fig. 2) and from four conventionally produced control milk samples using authentic reference compounds synthesized in the Laboratory of Organic Chemistry, University of Helsinki [28, 29]. Results of the analyses of milk samples are presented in Table 2. Organic skimmed milk contained 411 ± 65 ng/mL of equol. There was some equol (62 ± 16 ng/mL) in conventionally produced control milk samples. Furthermore, formononetin was also detected in organic milk samples but not in conventionally produced milk. Similarly, some daidzein was detected in organic milk samples, but due to the coelution of impurities we were not able to quantitate the amounts. There was no daidzein in conventionally produced milk samples. No genistein, biochanin A, or *O*-DMA was detected in either milk samples.

An equol concentration of 411 ng/mL (313–518 ng/mL) in Finnish organic milk is 40% higher than the highest

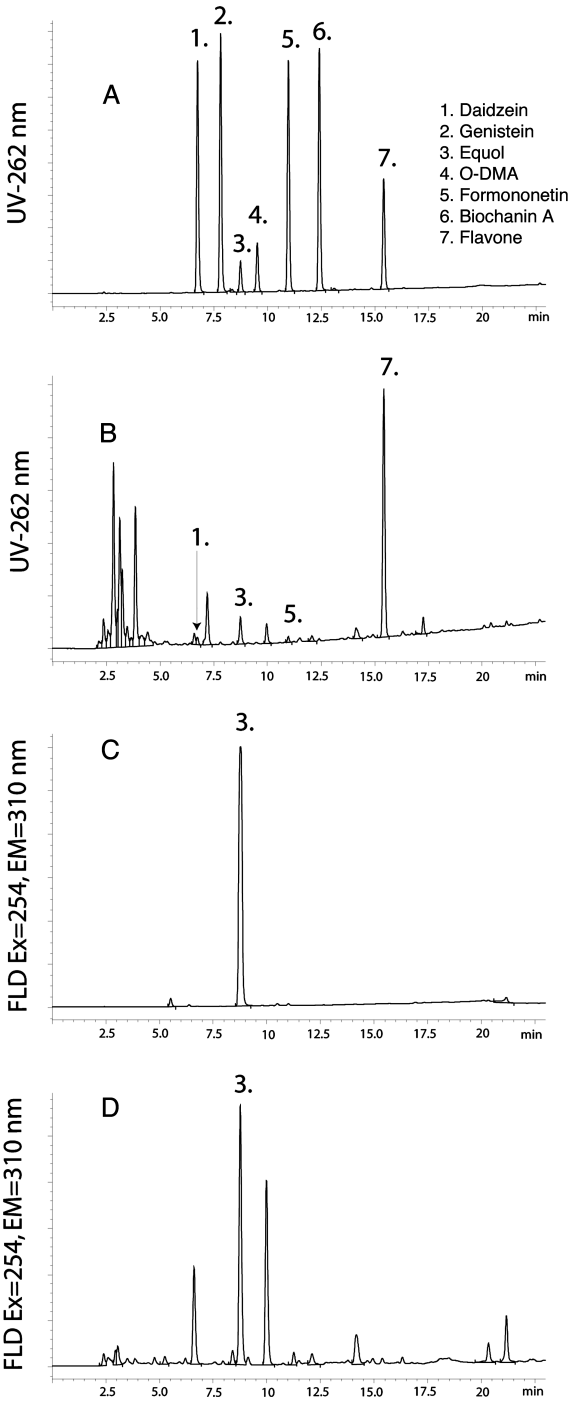

Figure 2. Typical HPLC UV and FL chromatograms of an organic skimmed milk sample (B, D) and authentic standards (A, C).

mean value reported in Australian milk (45–293 ng/mL). King *et al.* [21] collected bulk milk samples all over Australia, but highest equol values were found in Western Australia, where milk samples were on purpose collected during the spring when isoflavonoid concentration in the fields was expected to be maximal. When collecting milk sam-

Table 2. Isoflavone and metabolite concentrations in commercial organic and conventionally produced skimmed milk

Sample	Eq	Form
1	362.4 ± 21.1	d
2	419.5 ± 23.1	3.3 ± 0.9
3	373.4 ± 1.6	d
4	314.6 ± 14.7	d
5	313.3 ± 29.6	d
6	457.8 ± 28.4	4.6 ± 0.9
7	397.5 ± 25.3	3.9 ± 1.0
8	423.5 ± 11.8	4.2 ± 0.6
9	416.0 ± 8.9	4.7 ± 0.6
10	427.8 ± 10.0	4.1 ± 0.6
11	506.5 ± 33.5	5.1 ± 0.9
12	518.1 ± 64.0	5.7 ± 1.3
Mean ± SD	410.9 ± 64.7	4.5 ± 0.8
13[a)]	55.4 ± 3.9	nd
14[a)]	83.8 ± 10.9	nd
15[a)]	48.0 ± 5.5	nd
16[a)]	59.1 ± 7.0	nd
Mean ± SD	61.6 ± 15.5	

Values are in ng/mL; Form, formononetin; Eq, Equol. d, detected but below LOQ. nd below LOD.

a) Conventionally produced milk. Results are expressed as means ± SD. $p < 0.01$.

ples, farms that had clover cultivars with high isoflavonoid levels were selected if possible. Antignac *et al.* [23] have also analyzed bovine milk in France. They found 14–293 ng/mL of equol in commercial milk samples. The average equol value from organic milk was 191 ± 72 ng/mL but it is not clear how the animals in question were fed. In the French analyses, there is no real difference in the average equol content of skimmed as compared to full fat milk (68.9 or 72.0 ng/mL, respectively). High equol levels were only present in products from organic farms (average 191 ng/mL for nonskimmed milk but presumably comparable for skimmed organic milk). Even conventionally produced milk contained appreciable amounts of equol (average 36 ng/mL). It is interesting that in our study, average equol levels in Finnish organic skimmed milk were as high as 410 ng/mL, *i.e.*, more than double the French value. Similarly, equol concentration in conventionally produced Finnish skimmed milk samples was almost double compared to French values (62 ng/mL). Presumably, these differences reflect varying practices in cattle feed.

4 Concluding remarks

Finnish organic milk contains high levels of equol. Quite probably, this is due to the widespread use of red clover in organic farming to fix nitrogen in the fields. Red clover is known to contain considerable amounts of formononetin, which is converted to equol in the alimentary track of ruminant animals. Bovine milk is an important source of equol in food products. Many food products such as soy contain significantly higher amounts of equol precursors, but only less than half of the human population is capable of converting them into equol. The richest sources of equol in foodstuffs could be bovine blood products. The results from this short communication clearly show that further studies in this field are needed.

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