# Fresh and ensiled forages as a source of estrogenic equol in bovine milk: a review

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**ABSTRACT**: Isoflavan equol has recently been assessed in human nutrition as a potent estrogen with various health benefits. It is produced by intestinal microflora from isoflavones (IF) formononetin and daidzein. These dietary precursors are present particularly in soy products. However, only 20–35% of the Western adult population is capable of producing equol from the ingested IF. Cow's milk was proved to be an important source of equol produced from the IF in the rumen and intestines. Among forages, red clover (*Trifolium pratense*) is by far the most important source of both the IF. Their contents in fresh red clover forage are affected by variety and by several environmental factors. Leaves have the highest IF contents. Available data on changes in IF contents during wilting, drying, and ensiling are ambiguous. Content of equol in milk produced by dairy cows fed fresh or ensiled red clover can be at the level of several hundreds μg per litre.

**Keywords**: phytoestrogens; isoflavones; red clover; silage

Phytoestrogens represent a family of plant compounds showing both estrogenic and antiestrogenic properties. They may potentially confer health benefits related to cardiovascular diseases, cancer (particularly of breast and prostate), osteoporosis, and menopausal symptoms (Tham et al., 1998; Cornwell et al., 2004; Setchell and Cole, 2006; Andres et al., 2011; Jackson et al., 2011). As results from epidemiological studies, rates of these diseases are limited among populations with diets traditionally high in soy products rich in estrogenic compounds (Yuan et al., 2007).

Estrogenic activity has been shown in several groups of non-steroidal phytoestrogens with different chemical structure, chiefly in isoflavones, related coumestans, and in lignans. The estrogens occur in plants usually as glycosides, which are deconjugated by intestinal glycosidases. The released aglycones can then be further metabolized by the intestinal microflora.

The important biological activity was found for flavan equol (exactly: S-(–)-equol; 7-hydroxy-3-(4'-hydroxyphenyl)-chroman). Equol was firstly isolated from equine urine in 1932 and identified 50 years later in human urine as a metabolite of the soy isoflavones, daidzin and daidzein. It is produced by various intestinal bacteria from some ingested isoflavones (for a review see Setchell and Clerici (2010)). Dietary equol thus occurs in some foods of mammalian origin, with milk being its important source (Kuhnle et al., 2008).

Although almost every animal species studied till now produces equol when fed soy-containing diets, humans differ in that only 20–35% of the Western adult population is capable of producting equol from ingested soy or isoflavone supplements. A higher frequency of equol producers, approximately 50–55%, is found in adults living in Asian countries with regular and high consumption of soy products (Setchell and Cole, 2006). Thus,

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a great proportion of humans is not capable of producing endogenous equol and depends on its dietary intake.

For at least the latter group, bovine milk with high equol content may be desirable. Thus, a review of available data on factors affecting level of equol in cow's milk is needed.

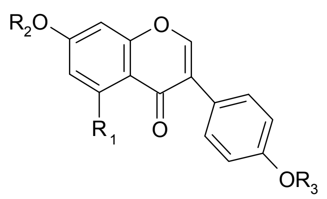
## Phytoestrogens

Various dietary phytoestrogens were detected in several hundreds of plant species. They exhibit weak estrogenic activity on the order of $10^{-2}$–$10^{-3}$ that of 17β-estradiol, but may be present in human body in concentrations 100-fold higher than the endogenous estrogens (Tham et al., 1998).

Coumestans, typical of lucerne (*Medicago sativa*), with coumestrol being the main compound, do not appear to be metabolized in the rumen and are partly carried out to cow's milk.

The major plant lignans, matairesinol and secoisolariciresinol, are converted by the intestinal bacteria to the mammalian lignans (called also enterolignans), enterolactone and enterodiol, respectively. Enterodiol can further be converted to enterolactone.

**Characteristics of isoflavones**. Chemical structures of main isoflavones are shown in Figure 1. They occur in plants preferably bound with β-D-glucose as glucosides daidzin, ononin, and genistin. The glucosides are readily hydrolyzed either by plant enzymes during mastication, or by acid of the stomach and by bacterial action in the gut.




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Daidzin | -H | -glucose | -H |
| Ononin | -H | -glucose | $-CH_3$ |
| Genistin | -OH | -glucose | -H |
| Daidzein | -H | -H | -H |
| Formononetin | -H | -H | $-CH_3$ |
| Genistein | -OH | -H | -H |
| Biochanin A | -OH | -H | $-CH_3$ |

Figure 1. Structures of the main isoflavones and their glucosides occurring in forages

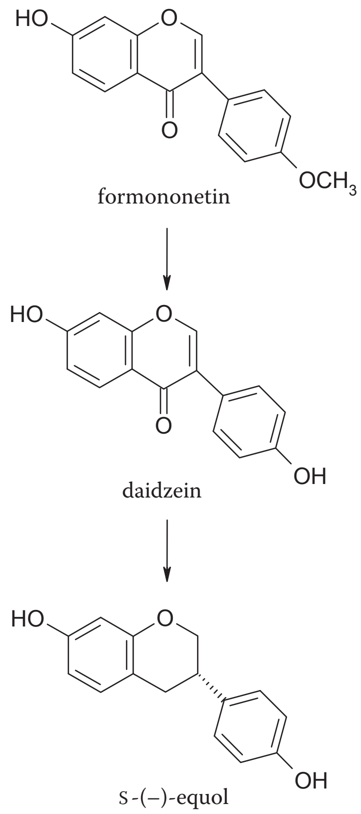



Figure 2. A scheme of the main metabolic route of formononetin and daidzein in the rumen

The released isoflavones are transformed in the rumen and the intestines by local microflora. While numerous participating bacteria have been isolated from human and rodent intestines (Setchell and Clerici, 2010), information on bovine rumen bacteria has been unique (Wang et al., 2005). Biochanin A and genistein are degraded into *p*-ethylphenol, a compound with no estrogenic activity. Formononetin is demethylated into daidzein and subsequently reduced into isoflavan equol. A scheme of the latter processes is shown in Figure 2. It gives only the major pattern, because also other products of the transformations were identified, namely *O*-demethylangolensin, angolensin, and 4'-*O*-methylequol.

## Isoflavones in forages

**Isoflavones in fresh forages**. Great infertility outbreaks of ewes grazing subterraneum clover (*Trifolium subterraneum*) in Australia in the 1940s initiated research on phytoestrogens occurrence also in related forage crops. The literature from that initial period was reviewed (Adams, 1989).

The main interest has been focused on red clover (*Trifolium pratense*) known to be weakly estrogenic in cows. Nevertheless, other legume forages were also tested. As reported Saloniemi et al. (1993, 1995), the estrogenic effect of lucerne was apparently caused by coumestrol, while the contents of formononetin and biochanin A were very low. Four tested varieties of white clover (*Trifolium repens*) contained very low levels of both isoflavones and coumestrol. Such low contents did not explain estrogenic effects observed in biological tests. The results proved Andersen et al. (2009b), who determined 11.4, 0.41, and 0.16 mg/g dry matter (DM) of formononetin in red clover, white clover, and lucerne, respectively. Only trace levels of phytoestrogens observed Sarelli et al. (2003) in birdsfoot trefoil (*Lotus corniculatus*).

Vetter (1995) quantified contents of four estrogenic isoflavones in leaves, stems, and flowers of seven clover species. The determined levels are considerably lower than values of other reports and can be thus adopted preferably as the comparative data. The results proved high isoflavone contents in subterranean clover and red clover and, moreover, in Alpine clover (*Trifolium alpestre*). On the contrary, *T. repens*, *T. montanum*, *T. fragiferum*, and *T. incarnatum* were low in these compounds. Data for three common *Trifolium* species are given in Table 1.

**Isoflavones in red clover**. Total content and composition of isoflavones in widely grown and fed red clover are affected by both genetic and environmental factors.

Schubiger and Lehmann (1994) compared formononetin content in 32 red clover varieties in Switzerland. Mean values and ranges were 7.7 (5.3–9.3) and 4.9 (3.1–6.3) mg/g DM in the first cut during May and in the third cut during August, respectively, both at the stage of starting flowering. Significant differences were observed among the varieties, with tetraploid being somewhat higher than diploid ones. The latter observation was supported by the results of Burda et al. (1997). However, Papadopoulos et al. (2006) found significant genetic variability for content of both total and individual isoflavones in 13 red clover varieties but these differences were not related to ploidy level (diploid vs. tetraploid).

Similar results were reported from Canada (Sivesind and Seguin, 2005). Average contents and range of the sum of formononetin and biochanin A in ten red clover varieties were 8.84 and 1.53–16.76 mg/g DM, respectively, with overall 55% formononetin, depending on variety, site, stand age, and harvest. Varieties had the most effect on isoflavone contents followed by the effect of sites. McMurray et al. (1986) reported decrease in formononetin content from 5.6 to 3.5 mg/g DM in first-cut red clover harvested between early May and mid-June under the conditions of North Ireland. The highest formononetin content in the following cuts was found in swards with the shortest regrowth period. The authors hypothesize that cooler temperatures in spring and autumn are likely to induce higher formononetin levels than warm days of mid-summer.

The decrease in formononetin content during red clover maturation from budding to flowering stage was reported also by Saloniemi et al. (1995) and Sarelli et al. (2003). Similar trend observed Kallela et al. (1987) in a mixture of red clover and timothy (*Phleum pratense*). The level of estrogenic compounds in timothy was negligible.

Du et al. (2012) determined the highest isoflavone yield per ha during the second year of red clover

Table 1. Mean contents (mg/g DM) of four isoflavones (total), formononetin, and daidzein in above-ground parts of three *Trifolium* species at flowering stage (adapted from Vetter, 1995)

| Species | Part | Total | Formononetin | Daidzein |
|---|---|---|---|---|
| Red clover *T. pratense* | leaves | 1.070 | 0.380 | 0.350 |
| | stems | 0.740 | 0.280 | 0.330 |
| | flowers | 1.210 | 0.390 | 0.460 |
| White clover *T. repens* | leaves | 0.027 | 0.007 | 0.005 |
| | stems | 0.119 | 0.085 | 0.009 |
| | flowers | 0.094 | 0.052 | 0.006 |
| Crimson clover *T. incarnatum* | leaves | 0.474 | 0.352 | 0.018 |
| | stems | 0.221 | 0.106 | 0.046 |
| | flowers | 0.164 | 0.112 | 0.017 |

Table 2. Mean contents (mg/g DM) of ten isoflavones (total), formononetin, and daidzein in above-ground parts of 13 red clover varieties at two growth stages (adapted from Tsao et al., 2006)

| Part | Total | | Formononetin | | Daidzein | |
|---|---|---|---|---|---|---|
| | EB | LF | EB | LF | EB | LF |
| Leaves | 20.39 | 27.78 | 8.22 | 11.09 | 0.60 | 0.08 |
| Stems | 17.34 | 12.08 | 11.71 | 7.43 | 0.14 | 0.34 |
| Petioles | 14.69 | 12.30 | 8.92 | 7.18 | 0.27 | 0.49 |
| Flowers | – | 2.38 | – | 0.80 | – | nd |

EB = early bud stage, LF = late flowering stage, nd = below limit of quantification

cultivation. Such research was induced by the use of isolated red clover isoflavones as nutraceuticals.

Isoflavones are distributed unevenly within the aerial parts of red clover. However, the literature data are ambiguous. Vetter (1995) reported comparable or higher contents in flowers than in leaves (Table 1). Booth et al. (2006) determined formononetin and daidzein contents in flower heads by one order of magnitude lower than in above-ground parts. The most thorough study was carried out by Tsao et al. (2006). They determined 10 isoflavones in parts of 13 red clover varieties. Selected results are shown in Table 2. Formononetin and biochanin A were the predominant isoflavones in all the varieties and all parts. The highest overall contents were in leaves, followed by stems, petioles, and flowers. Similar data were reported by Sivesind and Seguin (2005). Across stages of maturity, leaves were found to have the highest total isoflavone (formononetin + biochanin A) content followed by stems and inflorescences (11.97, 4.90, and 3.30 mg/g DM, respectively). Overall, the highest isoflavone contents were found in leaves and stems during the vegetative stages. Formononetin content declined until starting flowering, especially in stems.

An increasing level of soil phosphorus was reported to reduce formononetin content in red clover. For instance, the formononetin level was by 32% lower in plants cultivated in soil fertilized with 96 kg P per ha as compared with clover grown in soil with low P content (McMurray et al., 1986). Kallela et al. (1987) reported a strong positive correlation between sum of four isoflavones and crude protein contents in red clover. However, such information was not proved later.

**Isoflavones in silages**. The information on the effects of forage wilting prior to ensiling and ensiling process on the changes of isoflavones has been fragmentary. Ensilage of red clover is difficult in fresh state, wilting to about 35–40% DM prior to ensiling is therefore needed. Formic acid is the effective preservative if the effective wilting is impossible.

The results from 1960s and 1970s reported considerable increase of estrogenic activity during forage ensiling (Kallela, 1962, 1975). However, these data based on bioassays dealt with overall estrogenic activity caused by all present estrogenic compounds.

The contents of 0.53 and 0.013 mg/g fresh weight of formononetin and daidzein, respectively, were determined in silage of a mixture (1 : 1 w/w) of red clover and unspecified grass (Lundh et al., 1990). As reported by Sarelli et al. (2003), wilting of red clover from 25 to 40% DM decreased formononetin and daidzein contents by about 13 and 7–15% of the initial level, respectively. The contents of total four isoflavones (including genistein and biochanin A) in silage were by 18% higher than in the red clover prior to ensiling. The effect of tested silage additives, formic acid, and an inoculant containing *Lactobacillus plantarum* on total isoflavone changes was insignificant. Thus, growth stage and wilting were the factors of the greatest effects, while the effect of combined factors (growth stage × wilting × additive) was insignificant. Fresh birdsfoot trefoil and its silages contained only traces of isoflavones.

Sivesind and Seguin (2005) observed partly different effects of red clover preservation. They determined formononetin contents 9.02 ± 0.37, 7.22 ± 0.55, and 6.47 ± 0.16 mg/g DM in fresh, ensiled, and field-dried red clover, respectively. All the variants were prepared from the second harvest of the post-seeding year. Fresh red clover had DM of 28%, herbage for ensiling was wilted to 40% DM and ensiled for 50 days, and hay was produced at 90% DM after 2 days of field-curing. Thus, mean formononetin decrease was about 20 and 28% in silage and hay, respectively, as compared to fresh red clover.

Table 3. Mean contents (mg/g DM) of formononetin and daidzein in silages of red clover (adapted from Mustonen et al., 2009)

| Ensiled red clover | Formononetin | Daidzein |
|---|---|---|
| **Primary growth** | | |
| Early stage | 5.15 ± 0.30 | 0.16 ± 0.014 |
| Late stage | 2.95 ± 0.55 | 0.29 ± 0.033 |
| **Regrowth** | | |
| Early stage | 4.31 ± 0.18 | 0.17 ± 0.008 |
| Late stage | 6.47 ± 0.50 | 0.16 ± 0.017 |

Table 4. Mean contents (mg/g DM) of formononetin and daidzein in various silages (adapted from Höjer et al., 2012)

| Silage | Formononetin | Daidzein |
|---|---|---|
| Birdsfoot trefoil + timothy, 2nd cut | 0.14 ± 0.05 | 0.008 ± 0.007 |
| Red clover + timothy + meadow fescue, 2nd cut | 2.79 ± 0.26 | 0.024 ± 0.003 |
| Red clover + timothy + meadow fescue, 3rd cut | 2.97 ± 0.19 | 0.026 ± 0.003 |

Formononetin and daidzein contents in four types of unwilted red clover silages are given in Table 3. Unfortunately, changes during ensiling were not studied. Silages prepared from primary growth of a mixture of timothy-meadow fescue (*Festuca pratense*) contained no isoflavones (Mustonen et al., 2009). Also data of Höjer et al. (2012) prove that red clover is the main source of isoflavones in silages of legume-grass mixtures (Table 4).

Overall, available data indicate a decrease of both formononetin and daidzein during the ensiling process, particularly due to wilting.

## Isoflavones and equol in bovine plasma and milk

Phytoestrogens ingested by dairy cows may (*i*) be broken down into compounds with no estrogenic activity, (*ii*) pass through the rumen and intestines and be secreted in feces or urine or (*iii*) be transferred to the milk (Tucker et al., 2010).

**Isoflavones and equol in plasma of dairy cows**. Data on the level of isoflavones in cow's plasma have been scarce. Lundh et al. (1990) reported dynamics of the isoflavones in plasma following feeding red clover/grass silage. The maximum levels of both formononetin and daidzein were observed 1 h after feeding, while that of equol 2–3 h after feeding. Metabolic changes of equol precursors caused the delay of equol occurrence.

Mustonen et al. (2009) determined the isoflavones in pooled samples of blood taken before morning feeding with silages and 3 h thereafter. The contents of formononetin were 0.004–0.035 mg/l and traces in plasma of dairy cows fed with red clover silages and grass silages, respectively. The respective equol contents were 4.58–8.39 and 0.84–1.50 mg/l. Considerably lower mean contents of 0.014 and 0.018 mg/l of daidzein and equol, respectively, were reported by Krajčová et al. (2010) in plasma of cows fed with maize silage, lucerne hay, and concentrates.

**Isoflavones and equol in bovine milk.** The available data on isoflavones and equol in cow's milk are listed in Table 5. The data deal with farm tank milks, commercial milks, and milks from feeding experiments. While both formononetin and daidzein contents were mostly up to 10 μg/l, equol levels varied widely, mostly in tens and hundreds of μg/l. Red clover, both fresh and ensiled, was proved as the primary source of equol in milk.

Significantly higher equol contents in milk from organic than from conventional farms reported by Antignac et al. (2004) and Hoikkala et al. (2007) were caused by an elevated proportion of red clover in diets of organically fed dairy cows.

Recent results of feeding experiments with legume-grass silages (Table 6) concur that the apparent recovery of formononetin and daidzein to milk is very low, only about 0.1% of ingested amounts. Higher recovery, calculated as sum of daidzein and equol secreted into milk to sum of daidzein intake from feed, observed Krajčová et al. (2010). The ratio was 11.6 mg/g in cows fed maize silage, lucerne grass, and concentrates containing extruded rapeseed cake. Nevertheless, the level was only 1.4 mg/g if the rapeseed cake was substituted with extruded full-fat soy. Considerable amounts of plasma equol are apparently excreted in feces.

## Environmental impacts of isoflavones and equol

The United States Environmental Protection Agency has identified estrogens from animal feeding operations as a major environmental concern. The identified contamination of surface waters with various estrogens including isoflavones and

Table 5. Contents of equol and isoflavones (μg/l) in bovine milk

| Milk origin | Country | Main feed | Isoflavones equol | Isoflavones formo-nonetin | Isoflavones daidzein | Reference |
|---|---|---|---|---|---|---|
| **Farm tank milk** (76)* | | | | | | |
| Spring period | Australia | various including red clover | 293 ± 52 | – | – | King et al., 1998 |
| Summer period | Australia | various including red clover | 45 ± 10 | – | – | King et al., 1998 |
| **Commercial** | | | | | | |
| From conventional farms (19) | France | non-specified | 36.4 ± 14.8 | 0.3 ± 0.1 | 1.0 ± 1.4 | Antignac et al., 2004 |
| From organic farms (7) | France | non-specified | 191 ± 72.0 | 3.4 ± 1.0 | 3.9 ± 3.0 | Antignac et al., 2004 |
| Full fat milk (21) | France | non-specified | 72.0 ± 70.5 | 0.9 ± 1.3 | 1.2 ± 1.7 | Antignac et al., 2004 |
| Skimmed milk (4) | France | non-specified | 68.9 ± 68.7 | 1.1 ± 1.4 | 1.5 ± 1.8 | Antignac et al., 2004 |
| **Commercial skimmed** | | | | | | |
| From conventional farms (4) | Finland | non-specified | 61.6 ± 15.5 | nd | | Hoikkala et al., 2007 |
| From organic farms (12) | Finland | non-specified | 411 ± 64.7 | 4.5 ± 0.8 | | Hoikkala et al., 2007 |
| **Feeding experiments** | Denmark | red clover pasture | 215–355 | 4.0–6.3 | 1.1–1.9 | Andersen et al., 2009b |
| | Denmark | white clover pasture | 21.8–30.0 | 1.0–2.0 | 0.4–1.1 | Andersen et al., 2009b |
| | Denmark | lucerne pasture | 19.9–46.2 | 1.0–2.3 | 0.4 | Andersen et al., 2009b |
| | Norway | red clover/grass silage | 364 | 7.0 | 7.7 | Steinshamn et al., 2008 |
| | Norway | white clover/grass silage | 97.1 | 2.8 | 0.2 | Steinshamn et al., 2008 |
| | Denmark | grass-red/white clovers silage | 186 | 2.9 | 2.1 | Andersen et al., 2009a |
| | Denmark | lucerne silage | 3.0 | 2.1 | 0.8 | Andersen et al., 2009a |
| | Finland | red clover silage | 458–643 | – | – | Mustonen et al., 2009 |
| | Finland | grass (timothy and meadow fescue) silage | 171–287 | – | – | Mustonen et al., 2009 |
| | Czech Republic | maize silage + lucerne hay | 3.5 | – | 12.8 | Krajčová et al., 2010 |
| | Czech Republic | + extruded rapeseed cake or extruded full-fat soy | 55.5 | – | 15.8 | Krajčová et al., 2010 |
| | Sweden/Norway | birdsfoot trefoil/timothy silage, 2nd cut | 145 | 5.9 | 4.9 | Höjer et al., 2012 |
| | Sweden/Norway | red clover/timothy + meadow fescue silage, 2nd cut | 1494 | 12.6 | 16.0 | Höjer et al., 2012 |
| | Sweden/Norway | red clover/timothy + meadow fescue, 3rd cut | 1297 | 13.1 | 15.3 | Höjer et al., 2012 |

nd = content below limit of detection

*number of analyzed samples

equol initiated recent research on their sources and pathways of spreading.

Heifers fed red clover hay excreted in feces 1634, 163, 96.3, and 29.9 mg per day of equol, formononetin, daidzein, and genistein, respectively, while the respective values for heifers fed grass hay were 340, 18.3, 46.2, and 3.0 mg per day. The levels of the isoflavones and equol in urine were not affected by diet (Tucker et al., 2010).

Total isoflavone and equol loads in Swiss midland rivers were in the order of a few kg per year, and occurred mainly during summertime. Formononetin was the most frequent compound. Concentrations were usually in the lower ng/l range with the maximum of 524 and 217 ng/l for equol and formononetin, respectively (Hoerger et al., 2009). Among isoflavone cumulative loads in the catchments, annual values of 105–220, 0.5–1, and 0.1–5.1 kg/ha were determined for red clover, manure, and soil. In comparison, very low load of 0.0002 kg/ha per year via drainage waters were calculated. The isoflavones and equol can thus constitute a dominant portion of the total estrogenicity in small rural river catchments (Hoerger et al., 2011).

Table 6. Apparent recovery of formononetin + daidzein from various silages to milk (mg/g) in feeding experiments

| Silage | Recovery | Reference |
|---|---|---|
| White clover + grass | 1.20 | Steinshamn et al., 2008 |
| Red clover + grass | 0.21 | |
| Lucerne | 0.77 ± 0.035 | Andersen et al., 2009a |
| 1/3 lucerne + 2/3 maize | 0.30 ± 0.100 | |
| Grass + red clover | 1.23 ± 0.380 | |
| Birdsfoot trefoil + grass | 1.83 | Höjer et al., 2012 |
| 2nd cut red clover + grass | 1.07 | |
| 3rd cut red clover + grass | 0.96 | |

## CONCLUSION

Data from the literature show the possibility to produce cow's milk with equol content as high as 500 µg/l, or even higher, with feeding fresh or ensiled red clover. Such milk could be useful particularly for individuals lacking the ability to produce equol from its dietary precursors. Since only a proportion of formononetin is transformed to equol within cow's body, further research is needed on the selection of red clover varieties, and on optimization of cultivation, harvest, and preservation conditions aiming to elevate isoflavones content. Experimental comparison of the rate of equol formation from red clover and soy in bodies of dairy cows has been lacking until now.

Nevertheless, the effort for maximum level of equol in bovine milk has to be considered in broad links, such as position of red clover within crop rotation, its difficult ensilability, balance of feeding ration, potential elevated estrogenic effects of isoflavones on dairy cows, and increasing concern of the load of surface waters with estrogenic compounds.

## REFERENCES


Adams N.R. (1989): Phytoestrogens. In Cheeke P.R. (ed.): Toxicants of Plant Origin. Vol. IV. Phenolics. CRC Press, Boca Raton, USA, 23–51.

Andersen C., Weisbjerg M.R., Hansen-Møller J., Sejrsen K. (2009a): Effect of forage on the content of phytooestrogens in bovine milk. Animal, 3, 617–622.

Andersen C., Nielsen T.S., Purup S., Kristensen T., Eriksen J., Søegaard K., Sørensen J., Fretté X.C. (2009b): Phytooestrogens in herbage and milk from cows grazing white clover, red clover, lucerne or chicory-rich pastures. Animal, 3, 1189–1195.

Andres S., Abraham K., Appel K.A., Lampen A. (2011): Risks and benefits of dietary isoflavones for cancer. Critical Reviews in Toxicology, 41, 463–506.

Antignac J.-P., Cariou R., Le Bizec B., André F. (2004): New data regarding phytoestrogens content in bovine milk. Food Chemistry, 87, 275–281.

Booth N.L., Overk C.R., Yao P., Totura S., Deng Y., Hedayat A.S., Bolton J.L., Pauli G.F., Farnsworth N.R. (2006): Seasonal variation of red clover (*Trifolium pratense* L., Fabaceae) isoflavones and estrogenic activity. Journal of Agricultural and Food Chemistry, 54, 1277–1282.

Burda S., Ścibior H., Bawolski S. (1997): The effect of harvest term on isoflavone contents in red clover leaves. Acta Agrobotanica, 50, 87–92. (in Polish).

Cornwell T., Cohick W., Raskin I. (2004): Dietary phytoestrogens and health. Phytochemistry, 65, 995–1016.

Du W.H., Yue Y., Tian X.H. (2012): Variation of isoflavones production in red clover as related to environment, growth stage and year. Acta Alimentaria, 41, 211–220.

Hoerger C.C., Wettstein F.E., Hungerbuhler K., Bucheli T.D. (2009): Occurrence and origin of estrogenic isoflavones in Swiss river waters. Environmental Science and Technology, 43, 6151–6157.

Hoerger C.C., Wettstein F.E., Bachmann H.J., Hungerbuhler K., Bucheli T.D. (2011): Occurrence and mass balance of isoflavones on an experimental grassland field. Environmental Science and Technology, 45, 6752–6760.

Hoikkala A., Mustonen E., Saastamoinen I., Jokela T., Taponen J., Saloniemi H., Wähälä K. (2007): High levels of equol in organic skimmed Finnish cow milk. Molecular Nutrition and Food Research, 51, 782–786.

Höjer A., Adler S., Purup S., Hansen-Møller J., Martinsson K., Steinshamn H., Gustavsson A.-M. (2012): Effects of feeding dairy cows different legume-grass silages on milk phytoestrogen concentration. Journal of Dairy Science, 98, 4526–4540.

Jackson R.L., Greiwe J.S., Schwen R.J. (2011): Emerging evidence of the health benefits of S-equol, an estrogen receptor beta agonist. Nutrition Reviews, 69, 432–448.

Kallela K. (1962): Investigations on occurrence of plant oestrogens in Finnish AIV silage and hay, with special reference to red clover. International Journal of Fertility, 7, 358.

Kallela K. (1975): Effect of storage on estrogenic effect of red-clover silage. Nordisk Veterinaer Medicin, 27, 562–569.

Kallela K., Saastamoinen I., Huokuna E. (1987): Variations in the content of plant oestrogens in red clover-timothy-grass during the growing season. Acta Veterinaria Scandinavica, 28, 255–262.

King R.A., Mano M.M., Head R.J. (1998): Assessment of isoflavonoid concentrations in Australian bovine milk samples. Journal of Dairy Research, 65, 479–489.

Krajčová A., Schulzová V., Lojza J., Křížová L., Hajšlová J. (2010): Phytoestrogens in bovine plasma and milk – LC-MS/MS analysis. Czech Journal of Food Sciences, 28, 264–274.

Kuhnle G.G.C., Dell'Aquila C., Aspinall S.M., Runswick S.A., Mulligan A.A., Bingham S.A. (2008): Phytoestrogen content of foods of animal origin: dairy products, eggs, meat, fish, and seafood. Journal of Agricultural and Food Chemistry, 56, 10099–10104.

Lundh T.J.O., Pettersson H.I., Martinsson K.A. (1990): Comparative levels of free and conjugated plant estrogens in blood plasma of sheep and cattle fed estrogenic silage. Journal of Agricultural and Food Chemistry, 38, 1530–1534.

McMurray C.H., Laidlaw A.S., McElroy M. (1986): The effect of plant development and environment on formononetin concentration in red clover (*Trifolium pratense* L.). Journal of the Sciences of Food and Agriculture, 37, 333–340.

Mustonen E.A., Tuori M., Saastamoinen I., Taponen J., Wähälä K., Saloniemi H., Vanhatalo A. (2009): Equol in milk of dairy cows is derived from forage legumes such as red clover. British Journal of Nutrition, 102, 1552–1556.

Papadopoulos Y.A., Tsao R., McRae K.B., Mellish A.E., Fillmore S.A.E. (2006): Genetic variability of principal isoflavones in red clover. Canadian Journal of Plant Science, 86, 1345–1347.

Saloniemi H., Kallela K., Saastamoinen I. (1993): Study of the phytoestrogen content of goat's rue (*Galega orientalis*), alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*). Agricultural Sciences in Finland, 2, 517–524.

Saloniemi H., Wähälä K., Nykänen-Kurki P., Kallela K., Saastamoinen I. (1995): Phytoestrogen content and estrogenic effect of legume fodder. Proceedings of the Society for Experimental Biology and Medicine, 208, 13–17.

Sarelli L., Tuori M., Saastamoinen I., Syrjälä-Qvist L., Saloniemi H. (2003): Phytoestrogen content of birdsfoot trefoil and red clover: effects of growth stage and ensiling method. Acta Agriculturae Scandinavica, Section A, 53, 58–63.

Schubiger F.X., Lehmann J. (1994): Stoffe mit östrogener Wirkung in Rotkleesorten. Agrarforschung, 1, 361–364.

Setchell K.D.R., Clerici C. (2010): Equol: history, chemistry, and formation. Journal of Nutrition, 140, 1355S–1362S.

Setchell K.D.R., Cole S.J. (2006): Method of defining equol-producer status and its frequency among vegetarians. Journal of Nutrition, 136, 2188–2193.

Sivesind E., Seguin P. (2005): Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. Journal of Agricultural and Food Chemistry, 53, 6397–6402.

Steinshamn H., Purup S., Thuen E., Hansen-Møller J. (2008): Effect of clover-grass silages and concentrate supplementation on the content of phytoestrogens in dairy cow milk. Journal of Dairy Science, 91, 2715–2725.

Tham D.R., Gardner C.D., Haskell W.I. (1998): Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. Journal of Clinical Endocrinology and Metabolism, 83, 2223–2235.

Tsao R., Papadopoulos Y., Yang R., Young J.C., McRae K. (2006): Isoflavone profiles of red clovers and their distribution in different parts harvested at different growth stages. Journal of Agricultural and Food Chemistry, 54, 5797–5805.

Tucker H.A., Knowlton K.F., Meyer M.T., Khunjar W.O., Love N.G. (2010): Effect of diet on fecal and urinary estrogenic activity. Journal of Dairy Science, 93, 2088–2094.

Vetter J. (1995): Isoflavones in different parts of common *Trifolium* species. Journal of Agricultural and Food Chemistry, 43, 106–108.

Wang X.L., Shin K.H., Hur H.G., Kim S.I. (2005): Enhanced biosynthesis of dihydrodaidzein and dihydrogenistein by a newly isolated bovine rumen anaerobic bacterium. Journal of Biotechnology, 115, 261–269.

Yuan J.-P., Wang J.-H., Liu X. (2007): Metabolism of dietary soy isoflavones to equol by human intestinal microflora – implications for health. Molecular Nutrition and Food Research, 51, 765–781.

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