Variety, time of harvest and conditions during growing season have impact on red clover isoflavone content

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Red clover (*Trifolium pratense* L.) is the predominant legume used in northern European agriculture. Official red clover variety trials are conducted by Natural Resources Institute Finland (Luke) to determine the value of field crop varieties. The trials used for the current analysis were conducted in Luke units in southern Finland (Mikkeli) and northern Finland (Ruukki) in two consecutive years. Plant samples for isoflavone analyses were collected from four varieties grown as four replicates and harvested twice during both growing seasons. The four main isoflavones biochanin A, genistein, daidzein and formononetin were analysed using high performance liquid chromatography. Total phytoestrogen content in the varieties varied in the range of 11.2–14.8 mg g^{-1} dry matter (DM). The variety and the time of harvest had most effect on the isoflavone, especially formononetin, contents of red clover. A more northern growing area and challenging weather conditions were associated with increased isoflavone concentrations.

Key words: red clover, variety, isoflavone, daidzein, formononetin, equol

Introduction

There is growing interest in using forage legumes for ruminant feeding in organic as well as in conventional agriculture. In organic agriculture, inorganic N fertilizers are not permitted and legumes are used because of their ability to fix atmospheric nitrogen. Red clover (*Trifolium pratense* L.) is the major forage legume available for silage production in northern Europe. When legume plants are used, the need of N fertilizers diminishes in grassland based production systems (Halling et al. 2002, Halling et al. 2004, Vanhatalo et al. 2009).

Red clover is valuable ruminant feed for its crude protein and digestible fibre contents. In comparison with grass silage, red clover silage can stimulate higher dry matter intake and milk yield in dairy cows (Dewhurst et al. 2003, Bertilsson and Murphy 2003, Vanhatalo et al. 2006, Johansen et al. 2017). In addition, replacing grass silage with red clover silage can lead to desirable changes in milk fatty acid composition (Dewhurst et al. 2003, Bertilsson and Murphy 2003, Vanhatalo et al. 2006, Vanhatalo et al. 2007). Red clover diets promote growth and increase live weight gain in ewes and in lambs (Fraser et al. 2004, Moorby et al. 2004, Speijers et al. 2005, Graves et al. 2012).

Red clover contains isoflavones such as biochanin A, genistein, daidzein and formononetin (Kallela et al. 1988, Saloniemi et al. 1995, Sarelli et al. 2003). Isoflavones can have affinity towards oestrogen receptor, but the affinity varies greatly and in most cases high concentrations are needed (Pfitscher et al. 2008). In the rumen biochanin A is demethylated, first to genistein and thereafter to *para*-etylfenol and fenolacid, which do not have oestrogenic effects. Formononetin is converted first to daidzein and then to equol and its by-products. Physiologically the most important product of metabolism is equol (Shutt and Braden 1968, Cox and Braden 1974, Lundh et al. 1990). In addition, isoflavones have antioxidant capacity, for example genistein has been shown to inhibit significantly the replication of bovine herpes virus 1, which causes infectious bovine rhinotracheitis (Akula et al. 2002). Genistein also inhibited bovine viral diarrhoea virus (Lecot et al. 2005).

High intake on isoflavones, especially formononetin, can affect the fertility of sheep. Fertility problems in sheep are best known in Australia and New Zealand where sheep graze all year around (Adams 1995). In cattle, however, only very few case reports on possible fertility effects have been published (Thain 1966, Kallela et al. 1984). Sheep seem to be more susceptible to isoflavone phytoestrogens, even though metabolism of isoflavones is similar in sheep and cattle (Lundh 1990, Lundh et al. 1990).

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Intake of isoflavones affects milk composition. The first assessment of isoflavonoids in bovine milk was by King and others in Australia (King et al. 1998). They found the highest concentrations, 293 ng ml^{-1} equol, in samples from Western Australia, where prevalence of clover varieties is high. Similarly high equol concentrations were found in France (Antignac et al. 2003, Antignac et al. 2004). Recent research on the isoflavonoid concentrations in dairy milk associated with different silages and pasture has been done mainly in the Nordic countries (Purup et al. 2004, Hoikkala et al. 2007, Andersen et al. 2008, Steinshamn et al. 2008, Mustonen et al. 2009, Höjer et al. 2012).

The objective of this research was to study whether the variety, conditions during various growing seasons, growing site and time of harvest affect isoflavone content of red clover. It was hypothesized that isoflavone contents differ between the varieties and are higher in the northern rather than southern sites and that challenging weather conditions can increase the isoflavone concentrations of the varieties.

Materials and methods

General site description and management

According to the regulations of the Ministry of Agriculture and Forestry in Finland, the official variety trials are conducted by Natural Resources Institute Finland (Luke) to determine the value of field crop varieties (Anonymous 2004). The variety trials are performed in accordance with quality programme. During 2003 and 2004 variety trials for red clover were performed in two research sites, in Mikkeli (61°41'N, 27°12'E) and in Ruukki (64°41´N 25°04´E) (Kangas et al. 2010, Kangas et al. 2011). Four varieties of red clover Betty, Saija, Ilte and Jokioinen were studied in the official variety trials. The variety Bjursele was not included in the official trial; instead it was grown as border crop of the experimental fields. All four varieties were cultivated in four different plots at the two sites used. The first cut of the experimental plots and the border crop were harvested when five percent of inflorescences were visible (Järvi et al. 1998) in July (8–10 July 2003, 12–15 July 2004). The second cut were harvested at the end of August (22 August–1 September 2003) or at the beginning of September (2–7 September 2004). Two parallel samples from each red clover plots were collected. The variety tests were done during the 2003 growing season and repeated in 2004. Plant samples for isoflavone analyses were collected at the same time from the harvests as the samples for the official variety trial. Samples were stored frozen in –18 °C until analyses.

Weather at the sites

The length of growing seasons, the effective accumulated temperatures and precipitations for the years 2003 and 2004 at both sites Mikkeli and Ruukki are presented in the Table 1. The growing season was longer, amount of precipitation bigger and the effective accumulated temperatures lower during the year 2004 when compared to year 2003 (Hutila 2015).

Table 1. Weather at the sites

	Year	Beginning of the growing season[1]	End of the growing season[1]	Length of the growing season days	The effective accumulated temperature° C[2]	The accumulated precipitation mm[2]
Mikkeli Airport	2003	5th May	12th October	161	1283	377
Mikkeli Airport	2004	29th April	8th October	163	1247	389
Ruukki Siikajoki, Revonlahti	2003	5th May	11th October	160	1220	271
Ruukki Siikajoki, Revonlahti	2004	16th April	7th October	175	1126	451

[1]Effective day degrees; [2]During growing season

Analytical procedures

The quantitative high performance liquid chromatography (HPLC) analysis of herbage samples was presented in detail by Mustonen et al. (2006). In brief, 5 g of melted herbage was chopped and crushed and 25 ml of water was added. After shaking and incubation for 60 min at ambient temperature, 10 ml of 3.5 M hydrogen chloride (HCl) and 80 ml of ethanol were added to hydrolyse the isoflavone compounds. The mixture was shaken and heated to boiling and cooled. Hydrolysed samples were stored in a fridge at +6 °C. Extraction of herbage samples was done with 50 ml of ethanol and repeated three times by shaking for 1 min. The herbage sample, container and funnel were rinsed with 100 ml of ethanol and the combined ethanol extracts filtered through a Buechner funnel (filter paper Whatman 40). The extract was evaporated into a 20 ml volume and transferred into a 50 ml volumetric flask and filled to mark with ethanol. After 48–72 h storage in a fridge, the ethanolic herbage extract was filtered (0.45 µm GHP Acrodisc GF-filter) and dilutions were prepared into 10 ml volumetric flasks. Concentrations of external standards (genistein, daidzein, biochanin A, coumestrol and formononetin in ethanol) were 0.4–12.5 µg ml^{-1}. Each calibration curve used for quantification was characterized by a coefficient of determination (R^2) better than 0.999. Stock solution concentration was 25 µg ml^{-1}. Samples were analysed using two different HPLC detectors, ultraviolet (UV) visible and/or fluorescence. Isoflavones and metabolites were identified using authentic reference compounds. The analytical conditions were a linear gradient between 46% MeOH/water and 100% methanol for 23 min, post time 8 min, flow 1 ml/min, injection volume 10 µl. The eluent MeOH/aqueous trifluoroacetic acid was pH 3, UV detection at 262 nm and FLEX at 254 nm and EM 465 nm. Daidzein, genistein and biochanin A were analysed with UV and coumestrol and formononetin with fluorescence detector.

Statistical analyses

Experimental design was a completely randomized block design with four blocks (replicates) at both experimental sites. The herbage crop was harvested twice during summer and the trial was repeated the following year using the same blocks. Data collected were subjected to mixed model variance analysis, using the MIXED procedure of Statistical Analysis System (SAS Institute Inc, Cary, NC, USA) to identify significant treatment effects and interactions. Arithmetical mean values for isoflavones were calculated for both growing season (the years 2003 and 2004), for sites (Mikkeli and Ruukki), harvests (July and September) and varieties (Betty, Saija, Ilte and Jokioinen). The statistical model included year, experimental site, harvests and varieties and all their interactions. Replicate (year * experimental site) was a random factor in the model. Residuals for the entire data set were checked for normality and outliers using the UNIVARIATE procedure of SAS. The data comprised 128 observations for daidzein and genistein, but as for formononetin, biochanin A and total isoflavones two observations were considered outliers and excluded from the analysis. In the variance analysis, natural logarithmic data transformations were used. Post-anova comparisons between the treatments were made using a Tukey's test. Significant differences among treatment were considered at $p < 0.05$.

Results

The mean isoflavone concentrations of red clover as influenced by the growing year, experimental site, time of harvest and variety used are presented in Table 2, and results from the analysis of variance in Table 3. The main isoflavones in red clover varieties were formononetin, varying in range of 5.95–7.89 mg g^{-1} in DM, followed by biochanin A varying in range of 3.66–6.07 mg g^{-1} in DM. The respective concentrations of genistein, 0.49–0.55 mg g^{-1} in DM and daidzein, 0.23–0.30 mg g^{-1} in DM, were considerably smaller than the concentrations of the main isoflavones. No coumestrol was found from the samples. The total isoflavone content was as highest, 14.8 mg g^{-1} DM, in variety Ilte, which also had the highest formononetin concentration of all the varieties studied, 7.89 mg g^{-1} DM. Concentrations of total isoflavones including formononetin and biochanin A were significantly higher in northern than in southern Finland, and at the later (2. cut) than earlier (1. cut) harvest time as well as under the challenging growing conditions of the year 2004. This was evidenced with smaller effective accumulated temperatures and greater precipitation of the year 2004 in comparison to the previous year 2003.

Table 2. The mean isoflavone concentrations for red clover, mg g^{-1} dry matter. Natural logarithmic transformed means are presented in parentheses.

	Year		Experimental site		Harvest number		Variety[1]				SE
	2003	2004	South-Finland	North-Finland	H1	H2	Betty	Saija	Ilte	Jokioinen	
Daidzein	0.31 (-1.20)	0.23 (-1.51)	0.25 (-1.44)	0.30 (-1.27)	0.29 (-1.29)	0.25 (-1.42)	0.30 (-1.25)^{d}	0.23 (-1.52)^{c}	0.31 (-1.22)^{d}	0.25 (-1.43)^{c}	(0.18)
Genistein	0.51 (-0.70)	0.53 (-0.65)	0.52 (-0.67)	0.52 (-0.67)	0.54 (-0.63)	0.50 (-0.72)	0.55 (-0.61)^{b}	0.51 (-0.70)^{ab}	0.53 (-0.65)^{ab}	0.49 (-0.72)^{a}	(0.15)
Formononetin	6.26 (1.82)	7.40 (1.98)	6.60 (1.86)	7.06 (1.93)	5.92 (1.76)	7.74 (2.03)	7.15 (1.94)^{d}	5.95 (1.77)^{ac}	7.89 (2.05)^{e}	6.32 (1.83)^{bc}	(0.10)
Biochanin A	4.13 (1.38)	5.22 (1.61)	4.39 (1.44)	4.95 (1.55)	4.31 (1.41)	5.03 (1.58)	3.66 (1.26)^{c}	4.51 (1.48)^{d}	6.07 (1.77)^{e}	4.44 (1.46)^{d}	(0.17)
Total	11.2 (2.40)	13.4 (2.57)	11.7 (2.44)	12.8 (2.53)	11.1 (2.38)	13.5 (2.59)	11.6 (2.43)^{c}	11.2 (2.40)^{c}	14.8 (2.68)^{d}	11.5 (2.43)^{c}	(0.11)

[1]Superscripts indicate differences (^{a,b} p=0.05; ^{c,d,e} p<0.001) between the four red clover varieties; SE= standard error

Table 3. *p*-values from the analysis of variance of the isoflavone concentration of four red clover varieties grown for two years at two experimental sites and harvested twice during growing season

	Daidzein	Genistein	Formononetin	Biochanin A	Isoflavone total
Year	0.002	NS	<0.001	<0.001	<0.001
Experimental site	0.05	NS	0.002	0.006	0.001
Year x Site	NS	0.018	NS	0.02	NS
Variety	<0.001	0.023	<0.001	<0.001	<0.001
Harvest	<0.001	0.003	<0.001	<0.001	<0.001
Year × Harvest	<0.001	0.003	NS	NS	NS
Site × Harvest	0.002	NS	NS	0.001	0.027
Variety × Harvest	NS	NS	<0.001	0.007	0.005
Year × Variety × Harvest	NS	NS	0.016	NS	0.042
Site × Variety × Harvest	NS	0.027	NS	NS	NS

NS=non significant (p>0.05); Interactions for Year × Variety, Site × Variety, Year × Site × Variety, Year × Site × Harvest and Year × Site × Variety × Harvest were all non significant.

In the analysis of variance, there were significant year * variety * harvest interactions for total isoflavone (Fig. 1) and formononetin (Fig. 2) concentrations suggesting that increase in the isoflavone contents due to late cut owing to poor weather conditions in the year 2004 was greater with some varieties such as Betty than with other varieties. The same seemed to be true also for the border crop variety Bjursele although it was not included in the statistical analysis.

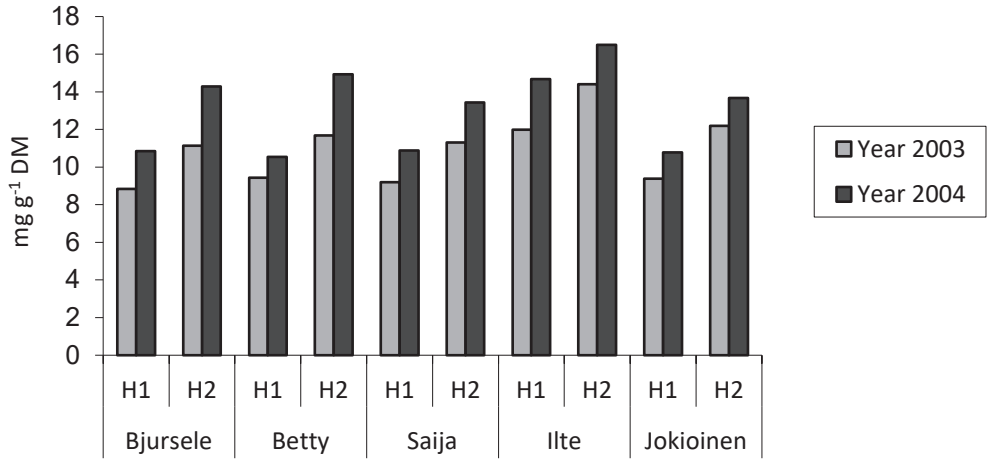

Fig. 1. The total isoflavone concentration (mg g^{-1} dry matter) for different red clover varieties at first (H1) and second (H2) harvest in 2003 and 2004

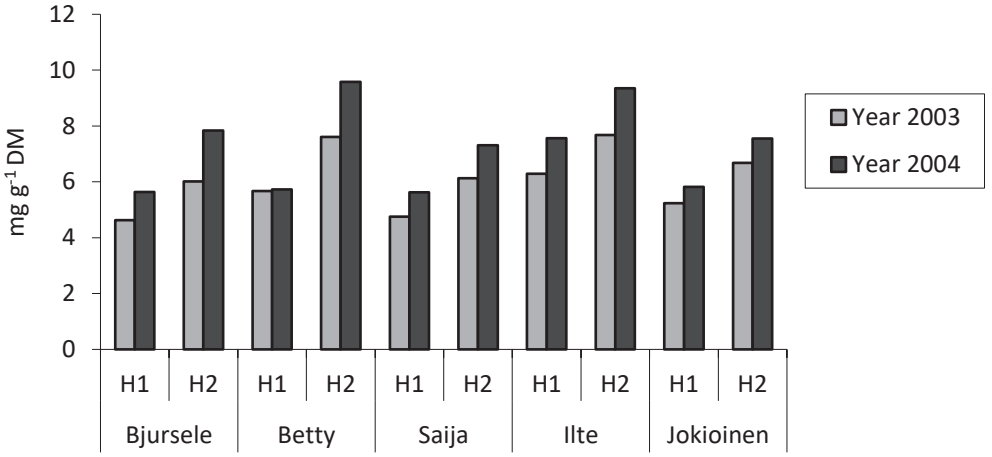

Fig. 2. The formononetin content (mg g^{-1} dry matter) for different red clover varieties at first (H1) and second (H2) harvest in 2003 and 2004

Discussion

The isoflavone contents found in this study are in line with those from previous studies, for example, total isoflavone content in Finnish red clover silages were at 10–25 mg g^{-1} in DM and formononetin content at 7–10 mg g^{-1} in DM (Kallela et al. 1988, Saloniemi et al. 1995). In the Nordic countries the total isoflavone content of red clover can range from 5–25 mg g^{-1} DM (Pettersson et al. 1984, Kallela et al. 1988, Saloniemi et al. 1995, Sarelli et al. 2003, Steinshamn et al. 2008, Andersen et al. 2009, Bernes et al. 2017). In previous studies it was established that isoflavone content is high in young crops, then declines during the middle of the growing season and rises again with advancing growth and can be even higher for later than earlier cuts (Kallela et al. 1988, Saloniemi et al. 1995). Moreover, it was reported that when the growing time is shortest, i.e. red clover is harvested again shortly after the previous cut, the formononetin content is highest (Mustonen et al. 2009).

Our results showing great variation in isoflavone content between the various varieties confirm results reported earlier. Red clover varieties differ significantly in isoflavone content, which means that genotype has an impact on isoflavone content (Kallela et al. 1988, Sivesind et al. 2005, Tsao et al. 2006, Saviranta et al. 2008, Bernes et al. 2017). Even if there are environmental effects like soil, stand age and harvest time, the variety choice will influence most on isoflavonoid amounts (Sivesind et al. 2005).

Temperature affects isoflavone levels, in a way that the lower the temperature the higher the formononetin content (McMurray et al. 1986). In an earlier study, higher isoflavone levels were detected in northern Finland compared with southern areas (Kallela et al. 1988), as recorded in this study between southeast Mikkeli and northwest Ruukki. There are also other factors that can affect isoflavone content. Nitrogen fertilizers can reduce the isoflavone content in swards (Kallela et al. 1987), but shortage of phosphate increased isoflavone amounts (Butler et al. 1967), especially formononetin content (McMurray et al. 1986). Elevated ozone levels, as well as biotic and abiotic stress factors, affect the roots by elevating isoflavone concentrations (Saviranta et al. 2008, Saviranta et al. 2010).

In agreement with our results the harvest time and season have influenced isoflavone content (McMurray et al. 1986, Kallela et al. 1987, Kallela et al. 1988, Sivesind et al. 2005, Booth et al. 2006). There is abundant production of isoflavones during spring and early summer when the growth of plants is fastest, and then production declines by midsummer (Kallela et al. 1980, McMurray et al. 1986,). In aftermath, the content increases and is high especially when compared with the age of the growth (Kallela et al. 1987). It has been shown that shorter growing periods of red clover resulted in higher silage formononetin content (Mustonen et al. 2009). In all parts of the plants formononetin diminishes as the plant matures. For the regrowth, the earlier the clover is harvested the higher are the isoflavone concentrations, and with the longest regrowth period the formononetin content is the lowest (McMurray et al. 1986).

Red clover leaves have the highest formononetin and biochanin A concentration; in stems and flowers formononetin predominates (Sivesind et al. 2005, Tsao et al. 2006, Saviranta et al 2008). Total levels of isoflavones are highest in leaves, intermediate in stems and lowest in flowers (Wu et al. 2003, Sivesind et al. 2005, Tsao et al. 2006). Just at the beginning of flowering the isoflavone content of inflorescences is highest (Sivesind et al. 2005) and declines after budding (Sarelli et al. 2003). For formononetin in leaves and stems, the highest concentrations were detected at the early maturity stage (Sivesind et al. 2005).

In conclusion, our results show that total isoflavone and formononetin concentrations of the present red clover varieties vary a great deal but are in range of concentrations reported previously. The variety and the time of harvest have the major effect on the isoflavone contents of red clover, but also the northerly growing areas and poor weather conditions can increase concentrations. Our results show that high and possibly harmful concentrations for sheep can be formed in northern location with poor weather and certain red clover varieties.

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