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Changes in the isoflavone concentration in red clover (*Trifolium pratense* L.) during ensiling and storage in laboratory-scale silos

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ABSTRACT

Isoflavones constitute one of the most common categories of nonsteroidal estrogen-like substances belonging to the broad group of phytoestrogens. The highest concentrations in the plant kingdom are found in the *Fabaceae* family. They have become a focus of research because of their estrogenic or anti-estrogenic effect and potential impact on human health. In recent years, several studies have focused on the impact of biotic and abiotic factors and farming management on the isoflavone concentration in plants and their impact on the composition of cow's milk. Nevertheless, knowledge about the effect of the ensiling process on isoflavone concentration remains limited. The objective of this work was to study the evolution of the concentrations of four compounds (daidzein, formononetin, genistein and biochanin A) in red clover (*Trifolium pratense* L.) ensiled at harvest. The impact of the field-drying process was also assessed. The plant material was ensiled in laboratory-scale silos using vacuum-packed plastic bags and stored over a 6-month period. The quality of the silages was checked throughout the experiment by determining the pH, lactic acid concentration, volatile fatty acids, crude proteins, cellulose and other chemical characteristics. The isoflavone concentration in fresh plant material was 2050, 1766, 306 and 127 μg/g DM for formononetin, biochanin A, genistein and daidzein, respectively. After 4 days of drying in the field, no significant change in isoflavone concentration was found, except for daidzein, which increased twofold. The laboratory-scale silos experiment, however, showed a decrease in isoflavone concentration during the first 2 weeks, followed by stabilization over the 5 remaining months. The concentrations fell by 26, 39, 66 and 73% for daidzein, genistein, biochanin A and formononetin, respectively. Animals fed with silage would therefore absorb fewer isoflavones than those fed with fresh plant material or hay.

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1. Introduction

Isoflavones are secondary plant metabolites and constitute one of the most common categories of phytoestrogens. They are structurally similar to 17β-estradiol, an endogenous steroid (Mostrom and Evans, 2012; Vitale et al., 2013; Ko, 2014). These nonsteroidal compounds have various functions in plants, such as attracting pollinators and seed-dispersing organisms. They also participate in plant defense mechanisms (Mostrom and Evans, 2012). Numerous human health benefits have been attributed to isoflavones (Mostrom and Evans, 2012; Vitale et al., 2013; Ko, 2014), but many of them are also considered to be endocrine disruptors, with the potential to cause health problems (Patisaul and Jefferson, 2010; Sirotkin and Harrath, 2014). These polyphenolic compounds are found mainly in *Fabaceae* (Mostrom and Evans, 2012). Among the forage plants of this family, red clover (*Trifolium pratense* L.) is known for its high concentrations of formononetin and biochanin A (Sivesind and Seguin, 2005; Tsao et al., 2006; Mostrom and Evans, 2012), but other isoflavones, as well as their glycosides forms (malonylglycoside and acetylglycoside), are also present (Sivesind and Seguin, 2005; Saviranta et al., 2010; Mostrom and Evans, 2012; Vitale et al., 2013). When ingested by ruminants, isoflavones are metabolized mainly by rumen micro-organisms and the main excretion route is through feces and urine, with only a small proportion being excreted in milk (Mostrom and Evans, 2012; Kalač, 2013; Njåstad et al., 2014). Formononetin is demethylated into daidzein and then reduced *via* hydrogenation and ring fission into equol; it can also metabolize into *O*-methyl equol or *O*-desmethylangolensin (Mostrom and Evans, 2012; Kalač, 2013; Njåstad et al., 2014). Equol is considered to be an antioxidant and could be used as a novel therapeutic agent against several diseases and cancers (Setchell and Clerici, 2010; Jackson et al., 2011; Mostrom and Evans, 2012). The two other microbial metabolites, however, appear to have low estrogenic activity (Setchell and Clerici, 2010; Jackson et al., 2011; Mostrom and Evans, 2012). Biochanin A is demethylated mainly into genistein and *via* ring cleavage into *para*-ethyl phenol (a compound that has no estrogenic activity) and organic acids (Mostrom and Evans, 2012; Kalač, 2013; Njåstad et al., 2014).

Several studies have been conducted recently on the phytoestrogen concentration in milk and on increasing the metabolites, especially equol (Kalač, 2013; Daems et al., 2016a). Animal feeding experiments have shown that a diet with a high isoflavone concentration (including daidzein and formononetin) greatly increases milk equol content (Kalač, 2013; Daems et al., 2016a). Milk from dairy cows therefore appears to be a potential source of equol in human diets (Kalač, 2011; Tsen et al., 2014). In order to increase the equol concentration in milk, it is necessary to feed cows with forages enriched with equol precursors (Kalač, 2013; Adler et al., 2015). The isoflavone concentration in plants is not constant and is affected by many factors, including abiotic factors, diseases, species, genotype, plant part and plants development stage (Sivesind and Seguin, 2005; Mostrom and Evans, 2012; Kalač, 2013). Post-harvest handling and conservation processes (such as silage) can also affect the isoflavone concentration in forages, but there is very little information on this subject. The effect of the ensiling process on isoflavone concentration is not well understood and there are contradictions in the available scientific literature. Sarelli et al. (2003) reported that the concentration of daidzein, genistein, formononetin and biochanin A in red clover silage was higher than that of raw materials, whereas Sivesind and Seguin (2005) found that formononetin concentration was lower in silage than in fresh red clover. The impact of the silage process on isoflavones therefore needs to be clarified.

Given that forages, specifically grass silage, constitute an important part of ruminant feeding in temperate regions, particularly when animals are kept indoors during winter (Huyghe et al., 2014), the study of isoflavone evolution during the silage process is relevant to the production of equol-enriched milk. Studying isoflavones in life-size silos would be a difficult task. Extended storage areas would be required and this could raise many analytical issues, such as sample homogenization, possible variation in the parameters studied between the different parts of the silage, and variability in grass composition in the grassland (Merry et al., 1995; Tsao et al., 2006; Kalač, 2013). Laboratory-scale silage systems seemed to be a good alternative. It was assumed that laboratory-scale silos would provide a reliable prediction of what was happening in farm-scale silos (Hoedtke and Zeymer, 2011; Cazzato et al., 2011). Two categories of laboratory-scale silos are commonly used: fixed-volume vessels (Cussen et al., 1995; Mustafa and Seguin, 2003; Palić et al., 2011) and vacuum-packed plastic bags (Sinnaeve et al., 1994; Johnson et al., 2005; Cherney et al., 2006; Hoedtke and Zeymer, 2011). Although the ensiling conditions are similar in the two techniques, the second one is increasingly preferred because it is easier to handle and enables the impact of packaging density to be studied and the fermentation gases to be analyzed (Johnson et al., 2005; Hoedtke and Zeyner, 2011).

The objective of the present work was to study the evolution of four isoflavones (daidzein, formononetin, genistein and biochanin A) in red clover silage over a 6-month period in laboratory-scale silos using a vacuum-packing system. The impact of the field-drying process on isoflavones was also investigated.

2. Material and methods

The experiment was conducted in autumn 2013 at the Walloon Agricultural Research Centre (CRA-W) in Gembloux, Belgium.

2.1. Experimental forage

The red clover (*T. pratense* L.) came from a mixed species grassland sown in spring 2010 after winter barley. The grassland was in the third year of production and was cut three or four times a year. The sown mixture consisted of red clover (*T.*