Phytoestrogens and Fatty Acids in Forage and Bovine Milk

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Cover: Red clover, timothy and grazing dairy cows
(photo: A. Höjer)

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Abstract

The overall aims of the studies underlying this thesis were to investigate effects of key factors on the concentrations and composition of phytoestrogens and fatty acids in forage species, and effects of differences in their concentrations and composition in forage on their concentrations in bovine milk.

In each of the spring cuts, the total isoflavone concentrations in red clover were higher at Umeå than at Skara. At Umeå, the concentration decreased with later cutting time, but at Skara there were no differences between the cutting times. The most abundant isoflavone found in red clover were formononetin, followed by biochanin A. In the summer cuts, the total isoflavone concentration decreased with a longer regrowth interval at Umeå, but this variable was not affected at Skara. Concentrations of each of the analysed isoflavones were considerably higher in red clover than in birdsfoot trefoil. Secoisolariciresinol was only found in birdsfoot trefoil.

Meadow fescue had in general higher concentrations fatty acids than timothy. The concentrations of all fatty acids in the grasses decreased with increasing lateness of cutting time in spring cuts. In general, concentrations of C18:0 and C18:1 were higher and concentrations of C16:0, C18:3 and total fatty acids lower in red clover than in birdsfoot trefoil. The concentration of almost all fatty acids decreased with later cutting time. There were correlations between leaf blade proportions, α-tocopherol, β-carotene and protein concentrations, and the fatty acid concentrations.

Two similar silage feeding experiments were conducted in which dairy cows were fed silage mixtures. In both experiments, the concentrations of all isoflavones were higher and those of mammalian lignans lower in milk from cows on the red clover-grass diets than in milk from cows on the birdsfoot trefoil-grass or white clover-grass diets. However, the largest observed difference in milk phytoestrogen contents was in the equol concentration.

For the fatty acids, the proportions in the milk and recoveries of C18:2n-6 and C18:3n-3 were higher on the red clover diets than on birdsfoot trefoil-grass or white clover-grass diets. Shortening the regrowth interval increased the proportion of unsaturated fatty acids in milk. Supplementation with α-tocopherol increased milk α-tocopherol concentrations but did not affect milk fatty acid composition.

Keywords: isoflavones, lignans, linoleic acid, α-linolenic acid, forage, ruminant

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Contents

List of Publications

This thesis is based on the work contained in the following papers, which are referred to by the corresponding Roman numerals in the text:

I Höjer, A., Nadeau, E. Purup, S., Hansen-Møller, J., and Gustavsson, A.-M. Phytoestrogens in red clover and birdsfoot trefoil as influenced by cutting time, site and year (manuscript).

II Höjer, A., Nadeau, E., Jensen, S. K. and Gustavsson, A.-M. Fatty acid concentrations in grasses and legumes in mixed leys as affected by cutting time, site and year (manuscript).

III Höjer, A., Adler, S., Purup, S., Hansen-Møller, J., Martinsson, K., Steinshamn, H. and Gustavsson, A.-M. (2012). Effects of feeding dairy cows different legume-grass silages on milk phytoestrogen concentration. *Journal of Dairy Science* 95(8), 4526-4540.

IV Höjer, A., Adler, S., Martinsson, K., Jensen, S. K., Steinshamn, H., Thuen, E. and Gustavsson, A.-M. (2012). Effect of legume-grass silages and α-tocopherol supplementation on fatty acid composition and α-tocopherol, β-carotene and retinol concentrations in organically produced bovine milk. *Livestock Science* 148(3), 268-281.

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The contributions of Annika Höjer to the papers appended to this thesis were as follows:

- I Planning the work jointly with co-authors, processing the data, responsible for writing the manuscript.
- II Planning the work jointly with co-authors, processing the data, responsible for writing the manuscript.
- III Planning the work jointly with co-authors, collecting the samples, performing some of the analyses, processing the data, responsible for writing the manuscript.
- IV Planning the work jointly with co-authors, collecting the samples, processing the data, responsible for writing the manuscript.

Abbreviations

B2	Birdsfoot trefoil-grass silage, two-cut system
BW	Body weight
CLA	Conjugated linoleic acid
DHL	High density lipoprotein cholesterol
DIM	Days in milk
DM	Dry matter
L3	Long-term ley silage
LDL	Low density lipoprotein cholesterol
NDF	Neutral detergent fibre
PPO	Polyphenol oxidase
R2	Red clover-grass silage, two-cut system
R3	Red clover-grass silage, three-cut system
S3	Short-term ley silage

1 Introduction

There is an increasing interest in the health effects of different food sources. Milk has been claimed for negative health effects due to, for examples, its high concentration of saturated fatty acids (Melnik, 2009) that are associated with increased concentrations of low density lipoprotein cholesterol (LDL, the 'bad' cholesterol) and increase risks for developing diabetes (FAO, 2010). However, milk also has health promoting substances, such as the bioactive components phytoestrogens (e.g. lignans and isoflavones), fatty acids (e.g. conjugated linoleic acids (CLA)) and vitamins (e.g. A and E). Phytoestrogens are associated with a reduced risk for cancers, such as breast, prostate and colon cancers, reduced risk for osteoporosis, and, maybe, also of cardiovascular diseases (Cornwell *et al.*, 2004; Peterson *et al.*, 2010). Some of the fatty acids in milk are claimed to be protective against cancers (Parodi, 1999) and there also are low concentrations of the essential fatty acids linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) in milk (Lindmark-Månsson, 2008; Doreau *et al.*, 2011). Concentrations of these substances in the milk can be influenced by the feeding of the cows. However, when increasing the proportion of unsaturated fatty acids in milk, risks for oxidation of the fatty acids increase, which increase the need for antioxidants, such as α-tocopherol and β-carotene, in the milk (Al-Mabruk *et al.*, 2004; Havemose *et al.*, 2006).

Forage is a source of both phytoestrogens and fatty acids to dairy cows. In this thesis, the effects of forage species, harvest system and site on the concentrations of phytoestrogens and fatty acids in fresh forage and their consecutive effects on the feed and milk are studied. To increase the understanding of the factors affecting these substances, a comprehensive introduction to this research area will follow.

1.1 Phytoestrogens

Phytoestrogens, the name deriving from *phyt*o (the Greek word for plant) and *estrogen*, the female hormone important for the oestrous cycle of humans and other mammals, are secondary, phenolic metabolites of plants.

The property shared by all phytoestrogens is the ability to induce or inhibit response of sex hormone receptors. In this respect, they share similarities with so-called endocrine-disrupting chemicals (EDCs), exogenous substances that can perturb the endocrine system and thus cause adverse health effects (Patisaul & Jefferson, 2010). Examples of man-made EDCs include: pesticides, such as DDT; industrial lubricants, such as PCBs; and plasticizers, such as phthalates and bisphenol A. However, in contrast to EDCs, most reported health effects of phytoestrogens on humans are beneficial (Jackson et al., 2011; Patisaul & Jefferson, 2010; Cotterchio *et al.*, 2008). Phytoestrogens are oestrogenic because of their similarity to 17β-oestradiol, which gives them the ability to bind to oestrogen receptors and thereby induce or inhibit downstream responses to the hormone.

Interest in phytoestrogens started in the 1940s, when severe problems with ewe fertility in Australia were reported (Bennetts *et al.*, 1946), caused by high concentrations of phytoestrogens, especially isoflavones, in subterranean clover (*Trifolium subterraneum* L.) the sheep consumed.

Phytoestrogens are divided into groups (for example isoflavones, lignans and coumestans) based on their chemical composition (Figure 1). The main focus of this thesis is on the isoflavones, but other phytoestrogens, mainly lignans, are also discussed.

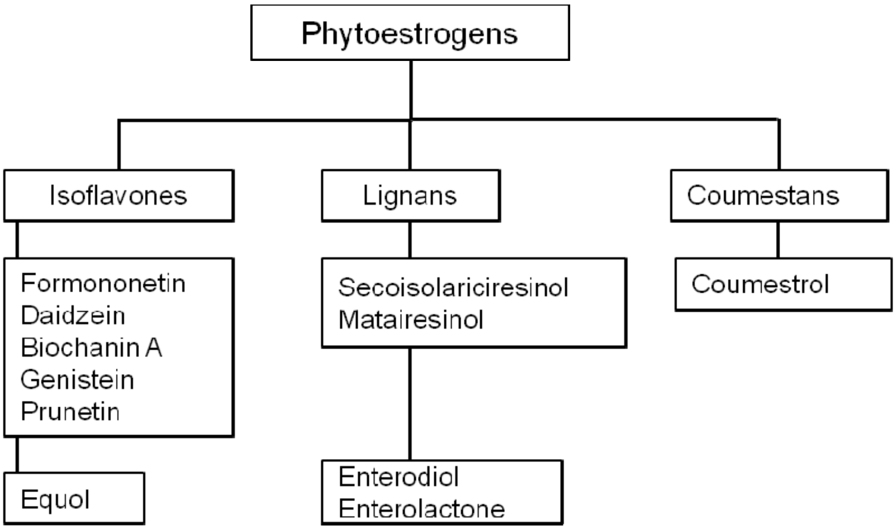

Figure 1. Examples of three classes of phytoestrogens: isoflavones, lignans and coumestans.

1.1.1 Synthesis and functions in plants

Isoflavones are members of a large group of substances called phenolic compounds, or simply phenolics, with a structure based on a phenol group; an aromatic ring with a hydroxyl functional group. The phenolics in plants are secondary metabolites with various functions, such as defence against herbivores or pathogens, protection against ultraviolet radiation and pollinator attraction (Taiz & Zeiger, 2010).

Isoflavones are synthesised from phenylalanine, which is produced via the shikimate pathway, and from malonic acid produced via the malonic acid pathway (Figure 2)(Taiz & Zeiger, 2010). In the shikimate pathway, simple carbohydrates are converted into three aromatic amino acids: phenylalanine, tryptophan and tyrosine. The phenylalanine is then metabolized to flavanoids (flavanones) through several steps together with malonic acid. The same pathway forms condensed tannins and other phenols, such as lignin. The flavanoids have two aromatic rings linked by a 3-carbon bridge and they can be further separated into four groups: anthocyanins, flavones, flavonols and isoflavones. A. The anthocyanins are pigments that help plants to attract pollinators and seed dispersers, while flavones and flavonols protect against ultraviolet radiation and may also attract pollinators. In addition, isoflavones, flavones and flavonols are important in interactions between legume roots and rhizobacteria in the soil. Isoflavones may also act as insecticides or pesticides, and some, e.g. those in legumes, may interfere with oestrogen receptors, because of their similarity to steroids (Taiz & Zeiger, 2010).

The metabolic pathway that generates isoflavones also produces lignans; dimers of monolignols derived from so-called hydroxycinnamic acids (*p*-coumaric, ferulic and sinapic acids) (Peterson *et al.*, 2010). When several monolignols are joined together, lignin is produced. Lignan and lignin should not be confused with each other, although they have similar structures.

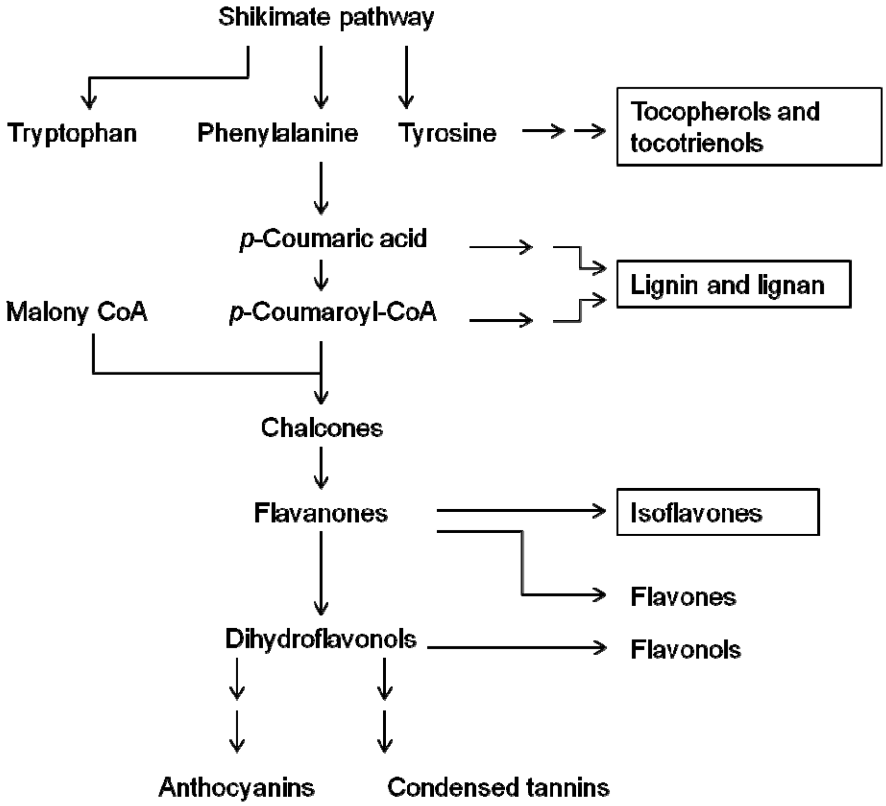

Figure 2. Schematic view of the biosynthetic pathway that produces lignans, isoflavone, tocopherol and tocotrienols (based on Asensi-Fabado & Munné-Bosch, 2010; Peterson *et al.,* 2010 and Taiz & Zeiger, 2010).

In plants, isoflavones are localized in the vacuole and their secretion through roots is an important step in nodulation (Dixon & Pasinetti, 2010). Many of the isoflavones and lignans are largely present as glycosides, with glucose or some other moieties, but they are only bioactive as aglycones, the unconjugated forms (Cederroth & Nef, 2009) (Figure 3). There are many naturally occurring isoflavones; 364 aglycones have been reported to date (Mazur & Adlercreutz, 1998).

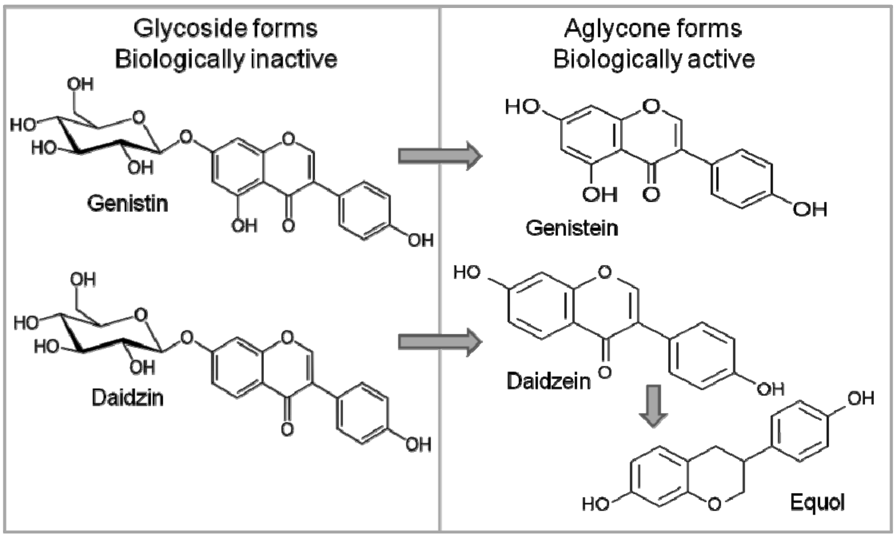

Figure 3. Glycoside and aglycone forms of the isoflavones genistein/genistein and daidzin/daidzein (based on Cederroth & Nef, 2009).

1.1.2 Phytoestrogens in feed

Phytoestrogens are present in both forage and concentrate/supplements of ruminant feed. For instance, red clover is known to have high concentrations of isoflavones, mainly formononetin and biochanin A (Sivesind & Seguin, 2005; Saviranta *et al.*, 2008). In red clover-grass silage and pasture the isoflavones daidzein, genistein, prunetin, and glycitein, the flavone chrysin and the flavanone naringenin have also been detected (Steinshamn *et al.*, 2008; Andersen *et al.*, 2009a). Low isoflavone concentrations have been reported in white clover (*Trifolium repens* L.) (Andersen *et al.*, 2009a; Zgorka, 2009) and Steinshamn *et al.* (2008) found lower concentrations of isoflavones, but higher concentrations of lignans and coumestrol, in white clover-grass silage than in red clover-grass silage. Pure birdsfoot trefoil (*Lotus corniculatus* L.) has low concentrations of formononetin and biochanin A (Sarelli *et al.*, 2003). Low or non-detectable concentrations of isoflavones have been reported in timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* Huds.) (Kallela *et al.*, 1987; Mustonen *et al.*, 2009).

There are also phytoestrogens in other species. One of the most intensively studied species, in this respect, is soybean (*Glycine max* L.), which has particularly high concentrations of genistein and daidzein; concentrations in soybeans and soy-containing foods of 20-160 mg/100 g DM have been reported (Mazur & Adlercreutz, 1998). Linseed (*Linum usitatissimum* L.) has high concentrations of lignans (Petit *et al.*, 2009) and lignans are also present in cereals, legumes and other vegetables (Peterson *et al.*, 2010). Lucerne

(*Medicago sativa* L.) reportedly contains higher concentrations of coumestrol, but lower concentrations of formononetin and biochanin A, than red clover (Moravcová *et al.*, 2004; Seguin *et al.*, 2004a; Andersen *et al.*, 2009a).

1.1.3 Factors affecting phytoestrogen concentrations in plants

Several factors affect concentrations of phytoestrogens in plants. First and foremost there are large differences between species, as previously stated. The effects of other factors have been mostly studied in red clover among the forage species, but also in other species to varying degrees.

Concentrations of isoflavones differ among parts of red clover plants. Tsao *et al.* (2006) found total isoflavone concentrations to be highest in leaves, followed by the stem, petiole and inflorescence. Saviranta *et al.* (2008) found the highest concentrations of daidzein and genistein in stems harvested in August and highest formononetin and biochanin A concentrations in young leaves harvested in June. Roots reportedly have almost as high formononetin concentration as leaves, but low concentrations of daidzein, genistein and biochanin A (Saviranta *et al.*, 2008). Concentration of phytoestrogens also vary among parts of lucerne plants, those of luteolin, quercetin and apigenin being higher in inflorescence than in stems and leaves (Seguin *et al.*, 2004a).

In red clover leaves, the total isoflavone concentration has been found to increase by about 30% between the early bud and late flowering stages, whereas in the stems and inflorescence the concentrations decrease with advancing maturity (Tsao *et al.*, 2006). In contrast, Sarelli *et al.* (2003) observed reductions in concentrations of isoflavones, especially formononetin and biochanin A, from the budding stage to flowering stage. Kallela *et al.* (1987) also recorded reductions in total isoflavone concentrations with advancing maturity of red clover, but maturity had minor effects on their concentrations in roots, according to Saviranta *et al.* (2008). In lucerne, effects of maturity have been detected in concentrations of coumestrol, apigenin, luteolin and quercetin, the coumestrol concentrations being lowest in the early flowering stage, when lucerne is usually harvested for use as animal forage (Seguin *et al.*, 2004a). Effect of season have also been observed, by Booth *et al.* (2006), who found the highest concentrations of daidzein and genistein in July and highest concentrations of formononetin and biochanin A in early September, in red clover harvested every two weeks during the growing season.

There are also differences in isoflavone concentrations between different cultivars of red clover (Tsao *et al.*, 2006; Saviranta *et al.*, 2008), but no significant differences in total concentrations of isoflavones between diploid and tetraploid red clover, according to Tsao *et al.* (2006).

In addition, Saviranta *et al.* (2008) found that red clover grown in greenhouses has higher concentrations than the same cultivars grown in the field. This might be due to differences in environmental conditions (e.g. light, temperature, nutrition) and/or differences in the density of the plants or the exposure to insects. Higher concentrations have also been reported in red clover grown in mixed swards in comparison with pure stands (Kallela *et al.*, 1987).

Differences in phytoestrogen concentrations between sites have also been recorded in lucerne and soybean (Seguin *et al.*, 2004b; Seguin & Zheng, 2006; Zhou *et al.*, 2011) and effects of photoperiod, day length and light quality on concentrations of flavonoids in some species, for example, bilberries (*Vaccinum myrtillus* L.) (Åkerström, 2010; Jaakola & Hohtola, 2010).

In soybean seeds, higher concentrations of isoflavones have been recorded in years with higher than average daily mean temperatures and drought stress (Seguin *et al.*, 2004b). However, Caldwell *et al.* (2005) found negative correlations between temperature and concentrations of phytoestrogens in dwarf soybean seeds grown in climate chambers, and drought stress increased the concentrations. Similarly, Tsukamoto *et al.* (1995) found that high temperature decreased concentrations of isoflavones in both whole soybean seed and (to a much lesser degree) their hypocotyls.

1.1.4 Metabolism in animals

As mentioned above, in plants phytoestrogens are largely present as glycosides, the biologically inactive form. However, when digested by ruminants, enzymes (glycosidases) in the plants, rumen or intestinal tract convert the glycosides into aglycones. In the rumen, biochanin A is demethylated by the microflora to genistein and then degraded to *p*-ethylphenol, a substance with no estrogenic activity because it cannot bind to oestrogen receptors (Pfitscher *et al.*, 2008), and dihydrogenistein (Mazur & Adlercreutz, 1998). Formononetin is demethylated to daidzein, which is subsequently converted to the isoflavan equol (Batterham *et al.*, 1965). These substances are then absorbed by the intestine. All cattle are believed to be able to produce equol from ingested precursors (Atkinson *et al.*, 2005), in contrast to humans, as only about 30 to 50% of individuals in Europe and North America carry gut bacteria capable of metabolizing formononetin and daidzein to equol (Rowland *et al.*, 2000). In some species (including humans, monkeys, chimpanzees and rats) formononetin and daidzein can be metabolized to *O*-desmethylangolensin, in addition to equol, as reviewed by Atkinson *et al.* (2005), but this metabolite has not been detected in bovine milk (Hoikkala *et al.*, 2007; Mustonen *et al.*, 2009).

In cattle and sheep, the isoflavones are re-conjugated in epithelial tissues of the rumen, reticulum, omasum and small intestine (Lundh, 1990). In the liver some conjugation also occurs, but at a slower rate (Lundh *et al.*, 1988). The conjugation detoxifies the phytoestrogens and makes them less reactive. Rates of conjugation are reportedly similar in the rumen, reticulum and omasum, but higher in the small intestines of bovines (Lundh, 1990). However, Lundh *et al.* (1990) found that the absorption of free and conjugated isoflavones from the rumen was very fast in cattle; plasma concentrations of formononetin and daidzein reached maximum levels within an hour of feeding them red clover-grass silage. In addition, much higher concentrations of equol than of formononetin and daidzein were observed in the cattle's plasma, but equol concentrations in the plasma were not affected by the feed intake, remaining constant during the entire sampling period.

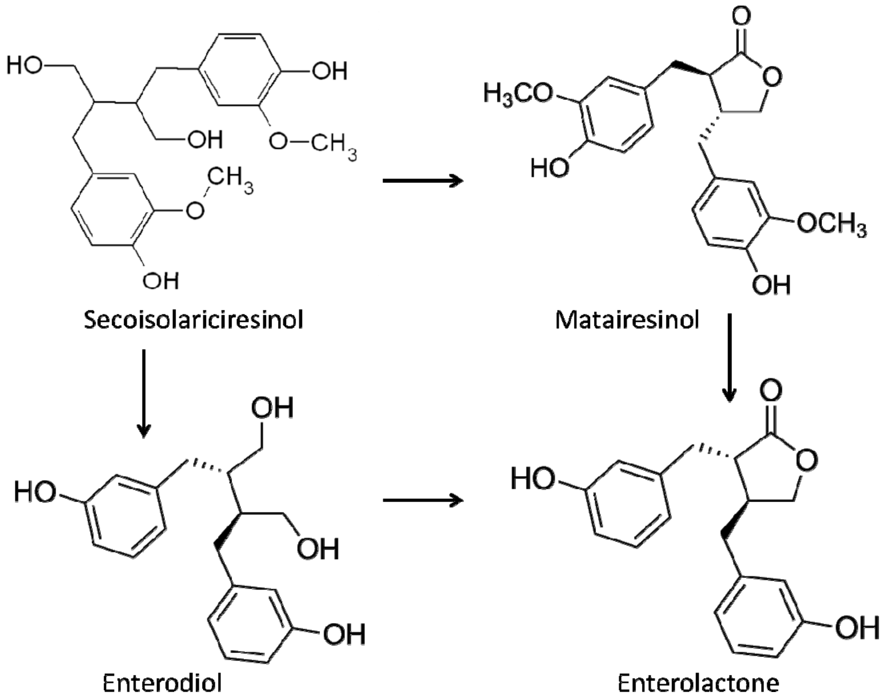

Figure 4. Metabolism pathways of lignans secoisolariciresinol, matairesinol, enterodiol and enterolactone (based on Heinonen *et al.* 2001).

Lignans in plants are present as both aglycones and glycosides (Peterson *et al.*, 2010). As for the isoflavones, gastrointestinal glycosidases release the attached glucose, or other moieties, yielding aglycones (Gagnon *et al.*, 2009). Then the intestinal bacteria metabolize some of the plant lignans to mammalian lignans, such as enterodiol and enterolactone (Gagnon *et al.*, 2009; Peterson *et al.*, 2010). The lignan matairesinol is mainly metabolized to enterolactone, whereas secoisolariciresinol is metabolized to enterodiol and enterolactone (Heinonen *et al.*, 2001) (Figure 4). However, other lignans are also precursors of enterodiol and enterolactone, for example pinoresinol, syringaresinol and lariciresinol (Heinonen *et al.*, 2001). Pinoresinol is metabolized to lariciresinol, which forms secoisolariciresinol (Heinonen *et al.*, 2001).

Phytoestrogens differ in their binding affinity to α and β oestrogen receptors (Cederroth & Nef, 2009). The isoflavone genistein has higher affinity than daidzein, and daidzein binds more strongly than biochanin A, which binds slightly more strongly than formononetin (Pfitscher *et al.*, 2008). All of these isoflavones bind more strongly to β receptors than to α receptors (Pfitscher *et al.*, 2008; Cederroth & Nef, 2009). Equol (as the S enantiomer, the form produced in humans and animals) has stronger binding affinity than its precursor daidzein, comparable to that of genistein (Muthyala *et al.*, 2004). Genistein and equol bind 24-fold less strongly than 17β-estradiol to oestrogen receptor β (Pfitscher *et al.*, 2008).

1.1.5 Effects on animals

Interest in phytoestrogens began when infertility problems in sheep were reported (Bennetts *et al.*, 1946), and found to be associated with ingested isoflavones found in subterranean clover, a widespread clover species in Western Australia appreciated for its ability to grow in typical infertile and nutrient-poor soils of agricultural parts of Western Australia (Adams, 1998). The clinical signs in affected ewes include (*inter alia*): prolaps of the uterus; mammary development and lactation in unmated ewes; and maternal dystocia, i.e. failure of the cervix to dilate and weak contractions of the uterus, especially in young ewes, leading to lambs dying in the uterus, as reviewed by Adams (1998). Wethers (castrated rams) are also affected (Adams, 1998).

Two types of infertility may occur: temporary and permanent (Adams, 1995). If the fertility is temporary, ewes fed feed containing phytoestrogens show decreased ovulation rates, but the symptoms disappear within 4 to 6 weeks after changing to a diet without phytoestrogens (Adams, 1995). If the ewes are continuously fed estrogenic feed the infertility may become permanent, because spermatozoa transport is impaired, although ovulation is not affected (Adams, 1998).

However, phytoestrogens do not only have negative effects. Moorby *et al.* (2004) found that lambs grazing red clover with high concentrations of formononetin had slightly higher daily weight gains than lambs grazing red clover with lower formononetin concentrations or perennial ryegrass, with the same daily DM intake in all three treatments (Moorby *et al.*, 2004). Similarly, Pace *et al.* (2011) found that raising lambs on subterranean clover with relatively low concentrations of phytoestrogens, especially formononetin, resulted in a higher daily weight gain, and had no negative effects on their reproductive systems or fertility.

Reports on the effects on cattle are not consistent. Heifers fed red clover silage by Austin *et al.* (1982) showed no clinical signs like those observed in sheep, and the pregnancy rate to first service was higher for heifers on red clover silage than on grass silage diets. However, negative symptoms, similar to those reported in sheep, have also been observed in cattle (Adler & Trainin, 1960; Kallela *et al.*, 1984).

1.1.6 Phytoestrogens in bovine milk

The ingested, absorbed and metabolized phytoestrogens are found in faeces, plasma and urine of cattle, and in their milk (Lundh *et al.*, 1990; Gagnon *et al.*, 2009; Tucker *et al.*, 2010). Phytoestrogens are also found in a wide range of other products of animal origin, such as eggs, meat, fish and seafood, with an average concentration of 20 µg/100 g wet weight (Kuhnle *et al.*, 2008).

One factor that reportedly affects milk phytoestrogen concentration is the botanical composition of the silage. Notably, Andersen *et al.* (2009b) found that feeding grass-clover (mixture of red and with clover) silage resulted in higher milk isoflavone and lower coumestrol concentrations than feeding lucerne or maize (*Zea mays* L.) silage, and Steinshamn *et al.* (2008) found that feeding red clover-grass silage resulted in higher isoflavone and lower lignan milk concentrations than white clover diets. The highest equol concentrations in milk have been reported when feeding red clover silages (Steinshamn *et al.*, 2008; Mustonen *et al.*, 2009). Higher formononetin, daidzein and equol concentrations have also been found in milk from cows grazing red clover-grass swards than when grazing white clover-, chicory- or lucerne-grass swards (Andersen *et al.*, 2009a). The cited authors also recorded lower enterolactone concentrations in milk from cows grazing red clover-grass swards than in milk from cows grazing lucerne- or chicory-grass swards. In addition Mustonen *et al.* (2009) found that increasing the regrowth interval of red clover used to make silage fed to cows increased equol concentrations in their milk Supplementing cows' diets with, for example, linseed may also increase mammalian lignan concentrations in milk (Gagnon *et al.*, 2009; Petit *et al.*, 2009).

There have been several national-level studies on phytoestrogen concentrations in bovine milk. King *et al.* (1998) recorded low concentrations of daidzein and genistein (< 5 µg/kg milk and at most 20-30 µg/kg milk, respectively) in milk from 76 farms in Australia, but higher equol concentration (45 to 293 µg/kg). In Finland, Hoikkala *et al.* (2007) found that commercial organic milk contained higher concentrations of equol than milk from conventional production (411 and 62 µg/kg, respectively). Traces of formononetin and daidzein were found in the organic milk but not in the conventionally produced milk. The differences were probably due to higher legume inclusion in the leys of the organic farms. Equol and enterolactone concentrations of 14-293 µg/kg and 14-94 µg/kg, respectively, have been recorded in commercial milk samples from French farms (Antignac *et al.*, 2004).

1.1.7 Importance of phytoestrogens for human health

Several attempts have been made to evaluate the health effects of phytoestrogens, and determine whether they are beneficial or adverse. Intake of phytoestrogens, especially lignans, has been associated with a reduced risk for breast cancer in over-weight women (Cotterchio *et al.*, 2008) and epidemiological studies indicate that dietary lignans decrease risks of coronary heart disease and cardiovascular disease (Peterson *et al.*, 2010). There are also indications that eating soy supplements can increase bone density, as reviewed by Jackson *et al.* (2011). Equol reportedly has health-promoting activities, including protection against prostate disorders, breast cancer, osteoporosis and cardiovascular diseases (Atkinson *et al.*, 2005; Jackson *et al.*, 2011; Lund *et al.*, 2011). In a study of 25,000 people living in the UK, no significant associations between phytoestrogen intake and the risk of breast cancer was found. However, the authors concluded that the intake of enterolactone and total lignans might affect the risk for colorectal cancer in women and that consumption of dairy products is associated with increased risk for prostate cancer in men (Ward *et al.*, 2010).

Several studies using cell lines or rats have investigated effects of phytoestrogens in milk on health and development. In one of these studies, Nielsen *et al.* (2012) found that applying whey from milk with different isoflavone concentrations had no effects on the proliferation and growth of human cancer cell lines, but all milk samples had an antioestrogenic effect on breast cancer cells.

1.2 Fatty acids

Fatty acids are a molecule consisting of a long carbon chain with a carboxyl group at one end. The carbon of the carboxyl group is designated carbon 1 and the other carbon atoms are sequentially numbered. The molecules are named according to both their total number of carbons and locations of double bonds, defined either by carbon numbers (e.g. 9, 12, 15-linolenic acid) or by counting from the last (omega or n) carbon in the chain, e.g. omega-3/n-3, omega-6/n-6, omega-9/n-9 (C18:3n-3) (Figure 5).

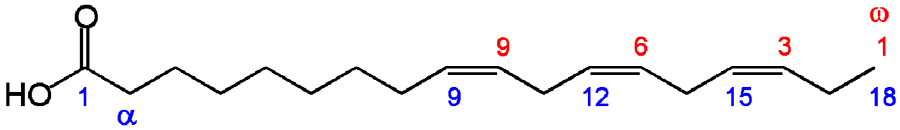

Figure 5. Numbering of the carbons in the fatty acid C18:3n-3 (α-linolenic acid).

When double bonds in a carbon chain are in *cis* configuration the chain is bent. However, when the double bounds are in *trans* configuration the chain stays straight. This affects the chemical properties of fat, as the straight chains of saturated and *trans*-fatty acids have higher melting points than the corresponding unsaturated fatty acids in *cis* configuration (Table 1).

Table 1. Names of some common fatty acids and their chemical composition (McDonald et al., 2002)

Trivial name	**Chemical name**	**Melting point (°C)**
Palmitic acid	C16:0	62.5
Stearic acid	C18:0	69.3
Oleic acid	*cis*-9 18:1	13.4
Vaccenic acid	*trans*-11 18:1	44
Linoleic acid	C18:2n-6	-7
Rumenic acid	*cis*-9, *trans*-11 18:2	20
α-Linolenic acid	C18:3n-3	-14.5

1.2.1 Fatty acid metabolism and functions in plants and animals

In plants, fatty acids are synthesised in the chloroplasts and other plastids by sequential elongation of acetyl-CoA with two carbons at a time (Taiz & Zeiger, 2010). Most of the fatty acids produced at this stage are C16:0 and C18:1, because of rapid desaturation of the synthesized C18:0. When the fatty acids are linked to glycerol to form glycerolipids, further desaturation of the fatty acids occurs resulting in, for example, C18:2n-6 and C18:3n-3. The composition of fatty acids in the membranes is important for several reasons. One is that it affects the plants' sensitivity to low temperatures, hence adjustment of the composition to environmental temperature changes may be critical (Taiz & Zeiger, 2010). Membrane lipids are also precursors of compounds that are used for signalling between plant cells, notably the plant hormone jasmonic acid is derived from C18:3n-3 and used (*inter alia*) to activate plant defences against insects and fungal pathogens.

In the chloroplasts, the main lipid components are glycolipids (with one or several glucose molecules), phospholipids and sulpholipids, in varying proportions (Hawke, 1973). In red clover and lucerne, for example, the main lipid components of the membranes of the chloroplasts are phospholipids, followed by monogalactosyl diglycerides and digalactosyl diglycerides with minor proportions of sulpholipids, sterols and sterol esters, while in grasses there are often more monogalactosyl diglycerides than phospholipids (Hawke, 1973).

In animals, fatty acids play important roles as energy stores, structural components of membranes and components of signalling molecules (Vance & Vance, 2002). As in plants, fatty acids in animal cells are formed from acyl-CoA by a circle-like enzymatic synthesis system, which adds two carbons to nascent chains in each “turn”. The initial products are predominately C16:0, together with smaller amounts of C14:0 and C18:0. In animal cells, phospholipids dominate in the membranes. The elongation of the fatty acids takes place in the endoplasmatic reticulum and mitochondria. The fluidity, permeability and stability of the membranes are affected by the fatty acid chain length, and the number and positions of the double bonds (Vance & Vance, 2002). There is substantial metabolism and rearrangement of fatty acids as the requirements for different fatty acids vary with development, growth and aging of the animal (Vance & Vance, 2002). In most animals, the liver and adipose tissues are the major sites of fatty acid synthesis.

Many required fatty acids can be synthesised by mammalian cells. However, animal cells cannot synthesize fatty acids with double bonds between carbon atom nine and the terminal methyl group. Thus, the fatty acids C18:2n-6 and C18:3n-3, which are essential, must be acquired from the diet. These fatty acids serve as precursors of biologically active eicosanoids (prostaglandins, leukotriens and tromboxanes), which are important for immune and inflammatory responses (McDonald *et al.*, 2002).

1.2.2 Factors affecting fatty acid concentrations and composition in plants

Total fatty acid concentrations vary among species. Reported concentrations include 14-30, 21-32, 19-40, 28-42 and 24-25 g/kg dry matter (DM) in timothy, meadow fescue, red clover, white clover and birdsfoot trefoil, respectively (Dewhurst *et al.*, 2001; Boufaïed *et al.*, 2003; Arvidsson, 2009; Van Ranst *et al.*, 2009b). Most of these fatty acids are unsaturated C18:3n-3 and C18:2n-6, and saturated C16:0 (Dewhurst *et al.*, 2001).

Several factors affect the fatty acid concentrations and composition in fresh herbage, and several further factors affect their concentrations in feed produced from it. In timothy, increasing maturity reportedly decreases concentrations of C16:0, C16:1, C18:2n-6, C18:3n-3 and, consequently, concentrations of total fatty acids (Boufaïed *et al.*, 2003). Concentrations of C18:2n-6, C18:3n-3 and total fatty acids are reportedly higher in spring growth than in summer growth, and the difference to be greater in grasses than in legumes (Boufaïed *et al.*, 2003). Elgersma *et al.* (2003) found that prolonging the regrowth interval reduced concentrations of total fatty acids in perennial ryegrass, had no effects on C18:0 and C18:2n-6 concentrations, and increased C18:1 concentrations. In another the same group found that it decreased concentrations of C16:1 and C18:3n-3, and affected the proportions of all major fatty acids in perennial ryegrass (Elgersma *et al.*, 2005). In addition, Dewhurst *et al.* (2001) reported that longer regrowth intervals decreased concentrations of C18:2n-6 and C18:3n-3 in perennial ryegrass.

Genetic differences between cultivars in the concentration and composition of fatty acids have been observed in perennial ryegrass (Elgersma *et al.*, 2003; Palladino *et al.*, 2009) and in timothy, meadow fescue, red clover and birdsfoot trefoil (Boufaïed *et al.*, 2003). Effects of N fertilization, including increases in the concentrations of several fatty acids, have also been recorded in both timothy (Boufaïed *et al.*, 2003) and perennial ryegrass (Elgersma *et al.*, 2005; Witkowska *et al.*, 2008).

Shading perennial ryegrass plants 24 h before harvest reportedly decreases their total fatty acid concentration (Dewhurst & King, 1998), whereas higher total fatty acid concentrations in perennial ryegrass have been recorded at lower solar radiation (Witkowska *et al.*, 2008). Changes in temperature mainly affect proportions of different classes of lipids, but changes in levels of individual fatty acids are small, according to Hawke (1973). However, a specific effect of temperature, observed by two groups, is an inverse relationship with the proportion of C18:3n-3 in plants grown under similar solar radiation regimes (Hawke, 1973; Witkowska *et al.*, 2008).

A management factor that is important for the fatty acid concentration in silage is wilting. Dewhurst and King (1998) found that wilting decreased the total fatty acid concentration, and the proportion of C18:3n-3, in perennial ryegrass. Wilting also reportedly reduces the concentration of fatty acids in timothy and in both red and white clover (Boufaïed *et al.*, 2003; Van Ranst *et al.*, 2009a). In contrast, Arvidsson *et al.* (2009a) found only minor effects of wilting on the fatty acid composition in timothy. However, in the cited study the wilt was shorter (less than 24 h) and the DM concentrations of the material were higher than in the studies where stronger effects of wilting were detected.

Conservation methods also affect fatty acid concentrations of the feed. Hay usually has lower concentrations of fatty acids than haylage, haylage has lower concentrations than silage, silage lower concentrations than fresh grass, and most of these differences appear to be due to the effects of prolonged wilting (Boufaïed *et al.*, 2003). However, the effects of different silage additives on the fatty acid concentration and composition are relatively small (Dewhurst & King, 1998; Boufaïed *et al.*, 2003; Arvidsson *et al.*, 2009a).

1.2.3 Fatty acid biohydrogenation in the rumen and metabolism

Most fatty acids that are present in ruminant feed are high in unsaturated fatty acids, but the fats of ruminant meat and milk are highly saturated due to biohydrogenation of fatty acids in the rumen, as described in several reviews.

In plants, most fatty acids are phospholipids, glycerolipids or other bound forms (Hawke, 1973). Lipolysis releases fatty acids from the glycerol in these forms, and is a prerequisite for biohydrogenation (Buccioni *et al.*, 2012). The lipolysis is mediated by either endogenous plant lipases or rumen microbial lipases (Buccioni *et al.*, 2012). *In vitro* studies of the plant enzyme polyphenol oxidase (PPO), present in, for example red clover, have found that lipolysis is reduced when PPO activity is high (Lee *et al.*, 2004; Lee *et al.*, 2007). However, Van Ranst *et al.* (2011) concluded that the importance of this inhibition of lipolysis in silage is questionable, since PPO-mediated inhibition of lipolysis failed to protect lipids from microbial lipases (which are important during later stages of ensiling and in the rumen). Instead, Van Ranst *et al.* (2011) postulated that polyunsaturated fatty acids could be protected against biohydrogenation through encapsulation in protein–phenol complexes.

In the rumen, unsaturated fatty acids are saturated by biohydrogenation (Figure 6). The bacteria involved in rumen biohydrogenation can be divided into two groups, A and B. Bacteria of group A hydrogenate C18:2n-6 and C18:3n-3 through several steps yielding *trans*-11 18:1 acid as the major end-product. Bacteria of group B mainly hydrogenate C18:1 fatty acids (*cis*-9, *cis*-11, *trans*-9 and *tran*s-11) to C18:0, but can also hydrogenate C18:2n-6 and C18:3n-3 (Kemp & Lander, 1984). *Butyrivibio fibrisolvens*, a common rumen bacterium, was one of the first bacteria found to biohydrogenate C18:2n-6 (Polan *et al.*, 1964).

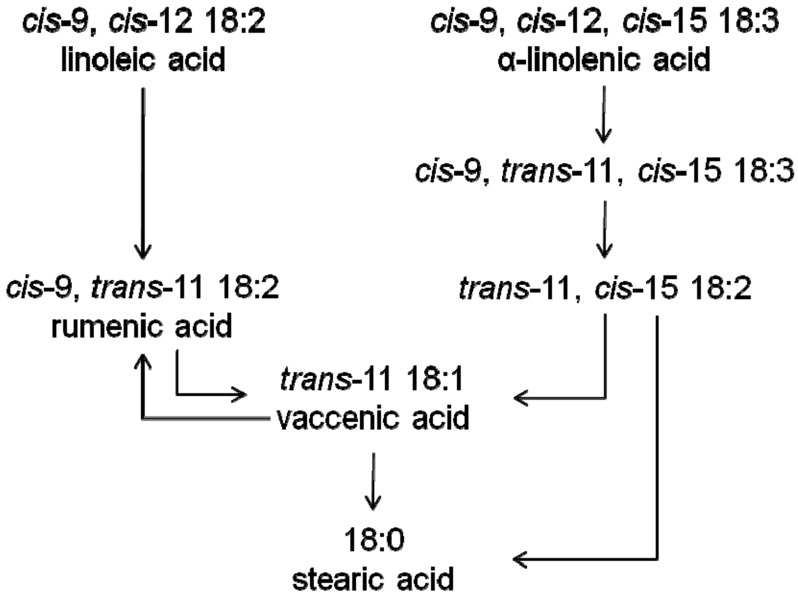

Figure 6. Some biohydrogenation pathways of linoleic and α-linolenic acid (adapted from Jenkins *et al.*, 2008).

The last step in the biohydrogenation, the synthesis of stearic acid (C18:0), is considered the rate-limiting step, as few bacteria are capable of catalysing this conversion (Harfoot & Hazlewood, 1988). Therefore, *trans*-11 18:1 tends to accumulate in the rumen. Most of the C18:2n-6 and C18:3n-3 fatty acids that enter the rumen are biohydrogenated, as only 6-23% of the these fatty acids that are ingested are recovered in the duodenal digesta (Dewhurst *et al.*, 2003a).

The fatty acids from the feed and those produced by biohydrogenation are not able to cross the rumen wall, in contrast to volatile short-chain fatty acids. These longer fatty acids from the feed are instead transported further to the mid or distal parts of the small intestine, where they are absorbed. Triacylglycerols are reconstituted from fatty acids in the intestinal mucous membrane then carried in the lymphatic system and into the blood. Triacylglycerols are united into droplets of fat, consisting of lipoproteins with a thin cover of phospholipids, cholesterol and protein (Lindmark-Månsson, 2008).

Biohydrogenation in the rumen results in the accumulation of *trans*-11 18:1, among other fatty acids (Buccioni *et al.*, 2012). In the udder and adipose tissue, there is an enzyme called Δ^9-desaturase, which introduces a *cis*-double bond at carbon 9 in the carbon chain of the fatty acid, yielding the conjugated linoleic acid (CLA) *cis*-9, *trans*-11 18:2 (Griinari *et al.*, 2000). It also desaturates C18:0 to *cis*-9 18:1, C16:0 to *cis*-9 16:1 and C14:0 into *cis*-9 14:1.

1.2.4 Fatty acids in bovine milk

About half of the fat in milk is synthesised in the udder, about 40% originates from fat in the feed and the remaining 10% from body reserves. In the udder short chain (4-10 carbon atoms) and medium chain (12-16 carbon atoms) fatty acids are synthesised from butyric and acetic acid in the blood (Lindmark-Månsson, 2008). The fat from feed consists predominantly of long chain fatty acids (longer than 18 carbon atoms), both saturated and unsaturated. The milk fatty acid composition is partly dependent on the composition of the fatty acids in the feed.

As reviewed by Dewhurst *et al.* (2006), higher concentrations of C18:2n-6 and C18:3n-3 have been found in milk from cows fed clover silage than in milk from cows fed grass silage. However, reported differences in effects of feeding red or white clover silage on milk C18:2n-6 and C18:3n-3 proportions are inconsistent. Dewhurst *et al.* (2003b) and van Dorland *et al.* (2008) found no differences in this respect between clover species (red or white) in silages. In contrast, Steinshamn & Thuen (2008) reported that milk proportions of both C18:2n-6 and C18:3n-3 were higher when cows were fed red clover silage than when they were fed white clover-grass silage. Feeding maize silage instead of grass silage, grass-clover silage, winter oat pasture or other pasture have been found to decrease proportions of polyunsaturated fatty acids (Dewhurst *et al.*, 2006; Kliem *et al.*, 2008).

In addition to the effects of forage species, effects of harvest date have also been reported. Vanhatalo *et al.* (2007) found higher proportions of total polyunsaturated fatty acids, and of C18:2n-6 and C18:3n-3, in milk from cows fed an early cut rather than a late cut of red clover silage.

Effects of conservation methods have also been reported. Notably, Dewhurst *et al.* (2006) found that feeding fresh herbage increased milk C18:3n-3 and CLA concentrations compared with feeding silage. Similarly Shingfield *et al.* (2005) found that feeding grass hay instead of grass silage increased milk C18:2n-6 and C18:3n-3 proportions, although the intake of those fatty acids was lower.

1.2.5 Health aspects of milk fatty acids

In humans, a minimum intake of C18:2n-6 equivalent to 2.5% of total energy intake is recommended, and up to 9% for beneficial effects. The recommended intake of C18:3n-3 is at least 0.5% of daily energy intake to prevent dietary deficiencies, and up to 2% can be eaten as part of a healthy diet (FAO, 2010).

Fatty acids with longer carbon chains and more double bonds can be synthesised from C18:2n-6 and C18:3n-3. Arachidonic acid (20:4n-6, AA) is synthesised from C18:2n-6, while eicosapentaenoic (20:5n-3, EPA), docosapentaenoic (22:5n-3, DPA) and docosahexaenoic (22:6n-3, DHA) acids are synthesised from C18:3n-3 (Livsmedelsverket, 2009). These long-chain fatty acids provide substrates for the production of eicosanoids; a group of substances important for regulation of blood clotting, blood pressure, smooth muscle contraction and the immune system (McDonald *et al.*, 2002). The recommended minimum intake of EPA and DHA is 0.25 g/day for adult males and non-pregnant/non-lactating adult females, rising to 0.3 g/day, for pregnant or lactating females, of which at least 0.2 g/d should be DHA (FAO, 2010). An increase of the intake of both eicosanoids formed from n-6 fatty acids and of n-3 eicosanoides has been proposed to be beneficial because of lowering risk factors for cardiovascular disease (FAO, 2010).

Mixtures of CLAs *trans*-9, *cis*-11 18:2, *trans*-9, *trans*-11 18:2 and *cis*-9, *trans*-11 18:2 and the fatty acid C18:2n-6 have been shown to inhibit growth of several human cancer cell lines (Parodi, 1999; Huang *et al.*, 2007). In milk, the main CLA is *cis*-9, *trans*-11 18:2, a fatty acid that only occurs in ruminant fats (Elgersma *et al.*, 2006). Another fatty acid that is specific to ruminants is the monounsaturated fatty acid *trans*-11 18:1.

Trans-fatty acids from industrially produced partly hydrogenated oils have been associated with several negative health effect, including increasing cardiovascular and coronary heart disease risk factors (FAO, 2010). However, in ruminant fat the proportion of *trans*-fatty acids (and consequently the human intake from this source) is very low. Therefore, there are no proven negative effects of *trans*-fatty acids from ruminant sources (Nishida & Uauy, 2009).

1.3 α-Tocopherol, β-carotene and retinol

A vitamin is an organic compound that is required by an organism as a vital nutrient in trace amounts (McDonald *et al.*, 2002). Vitamins may be either water soluble or fat soluble. Examples of fat soluble vitamins are vitamin A (retinol), vitamin D (ergocalciferol and cholecalciferol, which are also produced by ultraviolet radiation-mediated synthesis of sterols), and vitamin E (tocopherol/tocotrienol) (Ballet *et al.*, 2000).

Antioxidants are molecules that scavenge free radicals and/or inhibit the oxidation of other molecules, thereby preventing their formation and chain oxidation reactions that may cause damage or death to cells. Some substances are both vitamins and antioxidants, such as α-tocopherol, β-carotene and retinol.

1.3.1 α-Tocopherol

Vitamin E refers to a group of eight naturally occurring compounds (McDonald *et al.*, 2002). Four of these have a saturated side chain (tocopherols) (Figure 7) and the other four have an unsaturated side chain (tocotrienols) (McDonald *et al.*, 2002). Both tocopherols and tocotrienols are produced from metabolites that participate in carbohydrate metabolism, which are converted via the Shikimate pathway to chorismate and subsequently to tyrosine and, via several further metabolic steps, to tocopherol in the plastids (Figure 2). Tocopherols are located in the photosynthetic parts of plants, in the chloroplasts (Munné-Bosch & Alegre, 2002), thus leaves of forages species contain much more tocopherol than the stems (McDonald *et al.*, 2002). The most biologically active tocopherol is α-tocopherol.

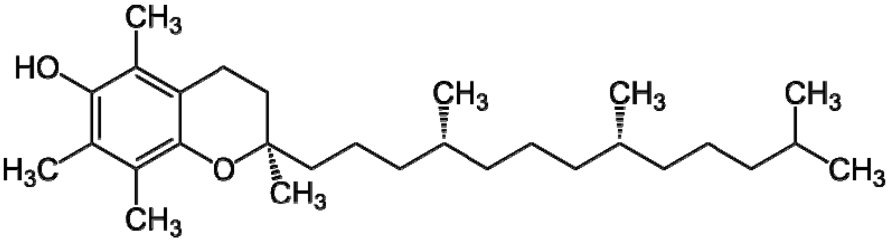

Figure 7. The chemical structure of α-tocopherol.

Tocopherols and tocotrienols act in two ways as antioxidants; the most important is as a scavenger of lipid peroxy radicals and the other is as a singlet oxygen reducer and chemical scavenger (Munné-Bosch & Alegre, 2002). Excessive concentrations of α-tocopherol may be harmful to the plant because it increases the rigidity of membranes. However, this may be counteracted by changes in the fatty acid profile of the membranes and elevated concentrations of β-carotene and other antioxidants (Munné-Bosch & Alegre, 2002). In ruminants, vitamin E acts together with another antioxidant, glutathione peroxidase. This is an enzyme containing selenium, hence selenium is also regarded as an antioxidant (McDonald *et al.*, 2002).

1.3.2 Carotenoids and retinol

Vitamin A, also called retinol, is an unsaturated monohydric alcohol (McDonald *et al.*, 2002). The precursors of the vitamin in plants are a few of a large group of substances called carotenoids, which can be divided into two subgroups: carotenes and xanthophylls. Most xanthophylls, such as lutein and zeaxanthin, cannot be converted to vitamin A, but the carotene β-carotene can be split into two parts, yielding retinol (Figure 8).

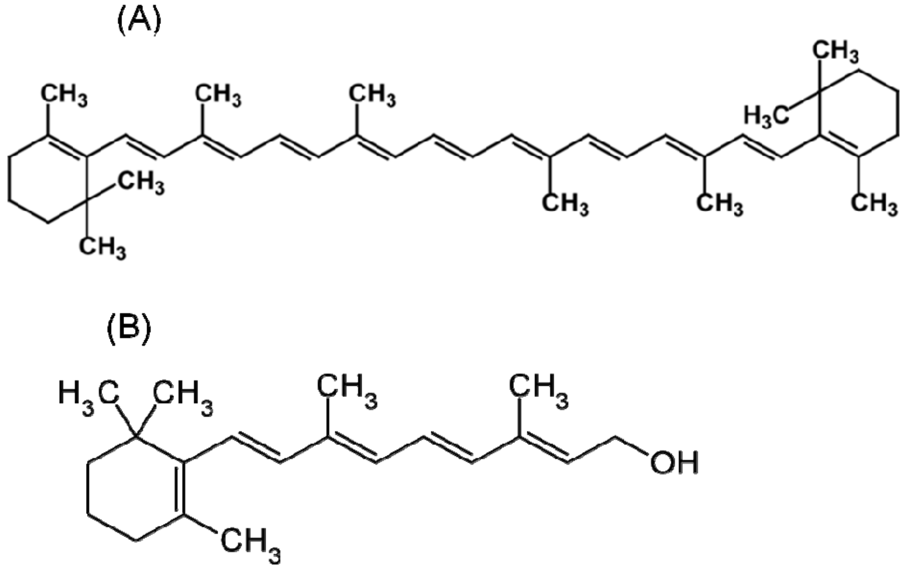

Figure 8. Chemical structures of (A) β-carotene and (B) retinol.

1.3.3 Factors affecting concentration in plants

Several factors affect concentrations of α-tocopherol and β-carotene in forage species. Notably, the concentrations differ between species. Lindqvist (2012) found higher concentrations of α-tocopherol in birdsfoot trefoil than in clover (white and red), and higher concentrations of β-carotene in birdsfoot trefoil and white clover than in red clover, over the growing season in a four-cut system. They also found higher concentrations of α-tocopherol in meadow fescue than in timothy, but no consistent differences over the growing season between timothy and perennial ryegrass. In addition, they found higher concentrations of α-tocopherol and β-carotene in birdsfoot trefoil-timothy silage than in red clover-grass silages. Higher concentrations of carotenoids have also been found in red clover than in perennial ryegrass (Nozière *et al.*, 2006a). However, the differences in carotenoids contents between legumes and grasses also seem to depend on maturity; in early stages they are more similar than at flowering, when legumes reportedly have higher concentrations than grasses (Ballet *et al.*, 2000). In both grasses and legumes, concentrations of β-carotene are higher in leaves than in stems (Ballet *et al.*, 2000).

Management factors also affect the concentrations. Wilting has been reported to reduce concentrations of α-tocopherol in birdsfoot trefoil-timothy mixtures, but not in red clover-grass mixtures (Lindqvist *et al.*, 2012). Wilting, especially outdoors with exposure to solar radiation, decreases carotenoid concentrations in forage, as reviewed by Nozière *et al.* (2006a).

The concentration of α-tocopherol has been reported to increase during ensiling of birdsfoot trefoil-timothy mixtures, both with and without an additive (Lindqvist *et al.*, 2012). However, the cited authors found higher concentrations of α-tocopherol in silage inoculated with bacteria and enzymes than in control or acid-treated silage of a red clover-grass (timothy or meadow fescue) mixture. The concentration of carotenoids reportedly decreases with ensiling, especially when oxygen is available due to delayed closure of the silos or when the pH is increased (Nozière *et al.*, 2006a).

1.3.4 Concentration in bovine milk

The concentrations of α-tocopherol, β-carotene and retinol in milk are partly dependent on their concentrations in the cows' feed, thus factors affecting their concentration in the feed also affect the milk concentrations (Nozière *et al.*, 2006a; Calderon *et al.*, 2007; Lindqvist *et al.*, 2011).

Several studies have found that forage conservation methods also affect milk concentrations of α-tocopherol and β-carotene; milk from cows fed silage diets reportedly has higher concentrations than milk resulting from hay diets (Shingfield *et al.*, 2005; Nozière *et al.*, 2006b). In addition, Shingfield *et al.* (2005) found that preserving silage by inoculation with lactic acid bacteria and an enzyme mixture (cellulase and hemicellulase) results in higher milk concentrations of α-tocopherol and β-carotene than adding formic acid to the silage.

The β-carotene concentration in milk also depends on breed, lactation stage, milk and milk fat yields, and possibly parity, as reviewed by Noizère *et al.* (2006a). In a study by Jensen *et al.* (1999), the concentrations of α-tocopherol, β-carotene and retinol in milk fat increased with lactation stage, but the amounts secreted into milk in mg per day were less strongly associated with the lactation stage. There also seems to be a maximal secretory capacity for these substances, and hence maximal level of daily secretion (Jensen *et al.*, 1999).

Supplementing cows' diets with α-tocopherol reportedly increases its concentrations in milk (Focant *et al.*, 1998; Givens *et al.*, 2003; Al-Mabruk *et al.*, 2004). However, effects supplements on milk α-tocopherol concentrations seem to depend partly on its intake from the basal diet (Lindqvist *et al.*, 2011).

2 Objectives

The overall aims of the studies underlying this thesis were to investigate effects of key factors on the concentrations and composition of phytoestrogens and fatty acids in forage species, and effects of differences in their concentrations and composition in forage on their concentrations in bovine milk. More specific objectives were to study:

- Effects of cutting time, site and year on the concentration and composition of phytoestrogens in two forage legumes grown in mixtures with grass during spring and summer.
- Effects of cutting time, site and year on the concentration and composition of fatty acids in two forage grasses and two forage legumes grown in mixed leys during spring, and correlations between α-tocopherol, β-carotene, crude protein concentrations and fatty acid concentrations.
- Effects of the botanical composition of forage, and harvest system, on the concentrations and composition of phytoestrogens in silage and milk when fed to dairy cows.
- Effects of silage selection and supplementation with semi-natural vitamin E on the composition of fatty acids and concentrations of fat soluble vitamins in milk when fed to dairy cows.

3 Materials and methods

3.1 Papers I and II

3.1.1 Experimental design, Paper I

In the study described in Paper I, two experiments were conducted, here designated Experiments 1 and 2. In the first a split plot design was applied to examine effects of site (using plots at two sites: Umeå and Skara) and cutting time (early, mid or late spring, 2005) on the phytoestrogen concentrations in red clover grown in a mixture with timothy in spring growth. The experiment also evaluated effects of site and year in combination, designated trial (assessed by analysing material cultivated at Umeå in 2005 and 2006, and at Skara in 2005), and cutting time (six weeks after the early cut and 6 or 8 weeks after the mid cuts in spring growth) on phytoestrogen concentrations of red clover in summer growth. The correlations between isoflavone and leaf blade proportion and crude proteins were also explored.

A completely randomized design was applied in Experiment 2, and in this experiment, differences in phytoestrogen concentrations between red clover and birdsfoot trefoil were studied by taking samples at the time of harvest of forage for silage production used in the Experiment 3 in Paper III (see below) during both spring and summer at Umeå 2008.

3.1.2 Experimental design, Paper II

In Paper II, samples were taken from Experiment 1. A split-split-plot experimental design was applied, with randomized complete blocks, to test effects of site in combination with year (again named trial), species and cutting time on forage concentrations of fatty acids. This was done using two field replicates of each of three forage species grown in mixtures (in sub-plots at each site), and variations in cutting time (in sub-sub-plots). Data obtained from the second-year ley at Umeå in 2006 and first-year ley at Umeå in 2005, for each of the forage species and cutting times, were also collected and compared. The species analysed were red clover and birdsfoot trefoil (both grown in mixtures with timothy), and timothy and meadow fescue (both grown in mixtures with red clover). The correlations between concentrations of fatty acids and α-tocopherol, β-carotene and leaf blade proportions were also explored.

3.1.3 Field management and harvests

The field experiments were located at Röbäcksdalen Research Centre, Swedish University of Agricultural Sciences, Umeå (63°45'N; 20°17'E; 5 m above sea level) and Lanna Research Station, Swedish University of Agricultural Sciences, Skara (58°21´N; 13°08´E; 75 m above sea level). Four forage species were sown in mixtures with barley as a cover crop: timothy (cv. Grindstad) and red clover (tetraploid cv. Betty and Sara at Umeå and Skara, respectively), meadow fescue (cv. Kasper) and red clover, and timothy and birdsfoot trefoil (cv. Oberhaunstaedter). The leys were established in 2004 and harvested in 2005 and 2006 at Umeå and in 2005 (but not 2006) at Skara. In 2006 a new field was established at Skara, which was harvested in 2007. The plot size in each case was approximately 1.5 x 13 m.

3.1.4 Samplings

The forage mixtures were each harvested at three maturity stages in spring growth. Targeted timings were: for the mid cuts, when timothy reached stage 50, as defined by Gustavsson (2011), i.e. when the first spikelet of the inflorescence is just visible above the flag leaf blade base; for the early cut, a week earlier than timothy was expected to reach stage 50; and for the late cut a week after this point. In addition, there were three cuts of summer growth: one six weeks after early spring growth, then two others, six and eight weeks after mid-spring growth.

At harvest, samples of the fresh matter were cut at stubble height and divided into various sub-samples. The sub-samples were sorted into: separate species to determine the botanical composition of the plots; leaves, petioles, stems and inflorescence to determine the leaf blade proportions; and phenological classes according to the scale devised by Gustavsson (2011) and Sanderson (1989) (Figure 9). These resulting fractions were dried in an air-forced oven. One other fraction was sorted into sown grasses and legumes and was frozen at -20°C within three hours of cutting. This fraction was used for analysis of phytoestrogens, fatty acids, α-tocopherol and β-carotene. Precipitation, temperature and solar radiation data for each of the sites during the growing seasons were obtained from the Swedish Meteorological and Hydrological Institute (SMHI).

Figure 9. Determination of phenology classes in red clover, from the second summer growth in Experiment 2.

3.1.5 Statistical analyses

In Paper I, the data obtained from Experiment 1 were analysed, using the PROC MIXED modelling procedure in SAS (2008), to assess effects of site (Umeå and Skara; n=2) and cutting time on phytoestrogen concentrations in the spring growth (early, mid or late; n=3). The data acquired for the summer growths were then analysed to assess effects of site and year (combined into a main effect named trial: Umeå 2005, Umeå 2006 and Skara 2005; n=3) and cutting time (6 weeks after early spring growth, and 6 and 8 weeks after mid-spring growths; n=3). Two field replicates were used for each cut. The relationships between leaf blade, crude protein, and phytoestrogen concentrations in the material harvested in Experiment 1 were analyzed using the REG procedure in SAS (2008). In Experiment 2, there were three replicates, except for the cuts in spring growth of birdsfoot trefoil (for which there were two replicates) and the acquired data were used to analyse the effect of species within cutting time.

In Paper II, the data acquired from Experiment 1 were analysed, using the PROC MIXED mixed modelling procedure in SAS (2008), to assess effects of site and year (combined into a main effect named trial; n=3), species (n=2) and cutting date (n=3) on fatty acid concentrations in first-year leys. Red clover was only analysed when grown in mixtures with timothy, timothy only when grown in mixtures with red clover, and grasses and legumes were analysed separately. In addition, first and second year leys at Umeå were compared (using data obtained from material harvested from the same plots in 2005 and 2006), to test effects of trial (n=2), species and cutting date (n=2). Relationships between fatty acid concentrations and crude protein, α-tocopherol and β-carotene concentrations in each of the species were also analysed, using the REG procedure in SAS (2008).

In all cases, differences between least squares means were evaluated with Tukey's test, and means were considered significantly different if $P < 0.05$ and to indicate a trend or tendency if $P < 0.10$.

3.2 Papers III and IV

Two similar silage feeding experiments were conducted (here designated Experiments 3 and 4), in which dairy cows were fed silage mixtures. Experiment 3 was conducted at Röbäcksdalen Research Centre, Swedish University of Agricultural Sciences, Umeå, Sweden and Experiment 4 at the Animal Production Experimental Centre, Norwegian University of Life Sciences, Ås, Norway. In both experiments, the effect of different silages on both phytoestrogen concentrations and composition, and the fatty acid composition and antioxidant concentrations in the cows' milk were studied. However, in the phytoestrogen study fewer cows' milk was analysed in Experiment 3. In Experiment 4 the effects of dietary supplementation with α-tocopherol were also studied.

3.2.1 Experimental design

In Experiment 3 a change-over Latin Square design based on Design No. 2 by Patterson and Lucas (1962) was applied. The design included three periods, each three weeks long, and three dietary treatments. Samples were taken at the end of each period, then all cows were switched to a new diet. Treatments included feeding dairy cows silage from two- or three-cut red clover-grass systems or a two-cut birdsfoot trefoil-grass system.

In Experiment 4, a change-over incomplete Latin Square design was applied, again with three 3-week periods but with four dietary treatments, sampling in the last week of each period. The treatments were applied in a 2x2 factorial design: short-term ley silage without α-tocopherol supplementation, short-term ley silage with α-tocopherol supplementation, long-term ley silage without α-tocopherol supplementation, and long-term ley silage with α-tocopherol supplementation.

3.2.2 Silage production

In Experiment 3, grass-legume systems were established in 2007 by sowing timothy cv. Grindstad, meadow fescue cv. Kasper and red clover cv. Betty (tetraploid Bjursele) in one field, and timothy cv. Grindstad and birdsfoot trefoil cv. Oberhaunstaedter in another field, with barley as cover crop. At establishment, the fields were fertilized with cattle slurry (30 Mg/ha). The timothy-meadow fescue-red clover field was divided into six strips and each strip was randomly assigned to harvesting as either a three-cut system or two-cut system. The birdsfoot trefoil-timothy field was harvested as a two-cut system. In all cases the spring cut was taken at the same date, when the blade base of the flag leaf was visible in 30% of timothy plants (on a dry-matter basis) according to Gustavsson (2011). Two cuts were also taken during the summer growth in the three-cut system, five and 10 weeks after the spring growth, respectively, and in the two-cut system a cut was taken during the summer growth seven and a half weeks after the spring cut.

Herbage from spring, early summer, late summer and second summer cuts was wilted, aiming to obtain a DM concentration of ca. 300 g/kg. The harvested herbages were preserved with an acid silage additive (ProMyr XR 630; Perstorp AB, Perstorp, Sweden) and the resulting silages were stored in six bunker silos. The diets were created as mixtures of these silages, with low proportions of spring growth material.

In Experiment 4, a long-term ley was established in 2003 with timothy cv. Grindstad, meadow fescue cv. Fure, perennial ryegrass cv. Napoleon and white clover cvs. Milkanova and Sonja, and fertilized with 25 Mg/ha cattle slurry. In addition, a short-term ley was established in 2007 with timothy cv. Grindstad and the diploid red clover cv. Bjursele, with barley as cover crop, and fertilized with 33 Mg/ha cattle slurry. A spring cut was taken at the booting stage of timothy, and two summer cuts were harvested seven and 15 weeks thereafter. The cuts were round-baled after 12-24 h of wilting and treated with an acid additive (GrasAAT N-Lacto; ADDCON Nordic AS, Porsgrunn, Norway). Diets were created as mixtures of the resulting silages, mixing one bale from each of the three cuts.

3.2.3 Animals and feeding

In Experiment 3, 24 multiparous Swedish Red dairy cows in mid lactation, with a mean initial daily energy-corrected milk yield (ECM)/day) at the start of the experiment of 28.9 kg — were blocked according to milk yield and DIM, and randomly allocated within block to the three dietary treatments. The treatments were feeding with three silage mixtures: red clover-grass silage from the two- or three-cut red clover-grass systems (designated R2 and R3, respectively), or the two-cut birdsfoot trefoil-grass system (B2). All cows were fed silage *ad libitum* through roughage intake control feeders (Figure 10) and received 5.4 kg DM concentrate/day. The cows were kept in a loose housing barn and milked twice a day at 0600 h and 1600 h.

Figure 10. A cow eating silage from the feeding system used in experiment 3, Umeå.

In Experiment 4, 16 multiparous Norwegian Red dairy cows in mid- lactation, with a mean initial milk yield at the start of the experiment of 27.5 kg ECM/day, were blocked according to sire, DIM and milk yield then randomly allocated within block to three of the four dietary treatments. The treatments were two silage mixtures in combination with and without α-tocopherol supplementation (2,400 IU/cow and day). The silage treatments were short-term ley silage (S3) and long-term ley silage (L3). All cows were offered silage *ad libitum* through roughage intake control-feeders. Concentrate was given separately at 5.4 kg DM per day. The cows were kept in a loose housing barn and milked twice a day at 0600 h and 1600 h.

3.2.4 Statistical analyses

Milk yield and feed intake were recorded daily throughout the experiments, and the data obtained from both experiments were analyzed using the MIXED model procedure implemented in SAS software (SAS, 2008). In experiment 1, milk from 15 cows was used for phytoestrogen analyses. The same statistical model was used for both experiments:

$$Y_{ijkl} = \mu + F_i + P_j + b_k + c_l + e_{ijkl}$$

where, Y is the response variable, μ is the mean, F is the fixed effect of diet (Exp. 3 i =1-3; Exp. 4 i = 1-4), P is the fixed effect of period (j = 1-3), b is the random effect of block (Exp. 3 k = 1-4 in Paper III and k = 1-8 in Paper IV; Exp. 4 k = 1-4), c is the random effect of cow (Exp. 3 l = 1-15 in Paper III and l = 1-24 in Paper IV; Exp. 4 l = 1-16) and e is the random residual error.

Differences between least squares means were estimated with Tukey's test, and means were considered significantly different if $P < 0.05$. In Exp. 4, contrasts were calculated for the effects of silage type and α-tocopherol supplementation.

3.3 Analyses

3.3.1 Phytoestrogens

Phytoestrogens in plants, feed and milk were analysed at Aarhus University, Research Centre Foulum, Denmark. Phytoestrogen concentrations in the feed and milk were analysed according to methods described by Steinshamn *et al.* (2008). The phytoestrogens analysed were the isoflavones formononetin, daidzein, biochanin A, genistein, and prunetin, the isoflavan equol, the coumestan coumestrol, and the lignans secoisolariciresinol, matairesinol, enterodiol, and enterolactone.

3.3.2 Fatty acids, α-tocopherol, β-carotene and retinol

Plant, feed and milk samples for determination of fatty acid concentrations were analysed at Aarhus University, Research Centre Foulum, Denmark. Plant and feed samples were extracted in a mixture of chloroform and methanol according to Bligh and Dyer (1959), with modifications described by Jensen (2008). The fatty acids in milk were also extracted according to Bligh & Dyer (1959), then analysed by gas chromatography as methyl esters (Jensen & Nielsen, 1996). The concentrations of α-tocopherol, β-carotene and retinol in the plants, feed and milk were analysed by high performance liquid chromatography after saponification and extraction in heptane (Jensen & Nielsen, 1996; Jensen *et al.*, 1999).

4 Results

4.1 Paper I

In each of the spring cuts, the total isoflavone concentrations were higher at Umeå in 2005 than at Skara in 2005. At Umeå, the concentration decreased with later cutting time, but at Skara there were no differences in this respect between the cutting times. The most abundant isoflavone found in red clover were formononetin, followed by biochanin A. Prunetin, daidzein and genistein were also found, but no lignans. In addition, the total isoflavone, formononetin, biochanin A, genistein and prunetin concentrations were all correlated with the leaf blade proportions in the spring cuts. In the spring cuts, the crude protein concentration was not correlated to any of the isoflavones.

In the summer cuts, the total isoflavone concentration decreased with a longer regrowth interval at Umeå in 2005 and 2006, but this variable was not affected by the regrowth interval at Skara. The concentration of formononetin decreased with a longer regrowth interval at Umeå in 2005 and 2006, but was not affected by the regrowth interval at Skara in 2005. The concentration of biochanin A decreased with longer regrowth interval at Umeå in 2005 and 2006, but increased at Skara. There were higher concentrations of total isoflavones in the cuts take eight weeks after mid-spring cuts at Skara in 2005 than in the corresponding cuts taken at Umeå in 2005, but no differences six weeks after the mid-spring cuts. In addition, there were higher concentrations in each of the summer cuts, at Umeå in 2005 than in 2006. The cutting time in the spring did not affect the total concentration of isoflavones or any of the individual isoflavones in summer cuts of red clover at either site. In the summer cuts, there were correlations between leaf blade proportions as well as crude protein, and total isoflavone, formononetin, biochanin A and genistein concentrations.

In Experiment 2, concentrations of each of the analyzed isoflavones, and total isoflavone concentrations, were considerably higher in the red clover than in the birdsfoot trefoil cultivated at Umeå in 2008. Increasing the regrowth interval before the first summer cut seemed to decrease concentrations of total isoflavones in both species. Matairesinol was not detected in any of the samples from either of the experiments. Secoisolariciresinol was only found in birdsfoot trefoil and prolonging the regrowth interval increased its concentration in the summer cut.

4.2 Paper II

The results from the study of the effects of cutting time, site and year on fatty acid concentrations in two forage grasses and two legumes grown in mixtures are presented in Paper II.

There were interactions between several of the factors, leading to, for example, larger differences between species in early cuts than in late cuts. Some of these interactions may have been due to differences in the developmental stage of the species among the trials.

Among the grasses, meadow fescue had higher concentrations of C16:0, C18:0 (in early cuts), C18:2 (in the early cut at Skara in 2007) and total fatty acids than timothy. The concentrations of C18:1 and C18:3 did not differ between timothy and meadow fescue. The concentrations of all fatty acids in the grasses decreased with increasing lateness of cutting time. Concentrations of the fatty acids C16:0, C18:0 (in early and mid-spring cuts), C18:2 and C18:3 were higher at Skara in 2005 than at Umeå in 2005. The concentration of C18:1, averaged over species, did not differ between the two trials. Concentrations of C16:0, C18:0, C18:2, C18:3 and total fatty acids were higher at Umeå in 2006 than at Umeå in 2005.

Among the legumes, concentrations of C18:0 and C18:1 were higher and concentrations of C16:0, C18:3 (at Skara in 2007) and total fatty acids lower in red clover than in birdsfoot trefoil. There was no difference between legumes in the concentration of C18:2. The concentration of all fatty acids except C18:1 decreased with later cutting time. There were higher concentrations of C18:1, but lower concentrations of C18:2n-6 at Umeå in 2005 than at Skara in 2005. In red clover cultivated at Umeå, the concentrations of C16:0, C18:1, C18:2 and C18:3 were higher in 2006 than in 2005, but the concentration of C18:0 did not differ between the trials.

In the grasses there were significant correlations between the leaf blade proportions, β-carotene and protein concentrations, and the total fatty acid concentration as well as for the concentrations of C16:0, C18:2 and C18:3. In meadow fescue, all the tested fatty acids were also correlated to α-tocopherol but in timothy there were correlations to total fatty acid concentration and concentrations of C18:2 and C18:3 but not C16:0.

In the legumes, fatty acids in red clover were correlated to leaf blade proportions, with the exception of C18:2, and to crude protein. None of the tested fatty acids in red clover were correlated to α-tocopherol or β-carotene. In birdsfoot trefoil, there was a tendency for significant correlations between leaf blade proportions and significant correlations for α-tocopherol, and the concentrations of C18:2, C18:3 and total fatty acid concentrations.

4.3 Paper III

The results presented in Paper III showed clear effects of the species used to produce silage on the concentration of phytoestrogens in the cow's milk. In both experiments, there were substantially higher concentrations of all detected isoflavones in the red clover-grass silage diets than in both the birdsfoot trefoil-grass and white clover-grass silage diets. The main contributors to isoflavones in the cows' intake were formononetin and biochanin A in the silages, and the total isoflavone intake was 4.0, 70.0 and 70.5 g/day, for diets B2, R2 and R3, respectively, in Experiment 3, and 75.3 and 3.9 g/day for diets S3 and L3, respectively, in Experiment 4.

In both experiments, the concentrations of all isoflavones were higher in milk from cows on the red clover-grass diets than in milk from cows on the birdsfoot trefoil-grass or white clover-grass diets. However, the largest observed difference in milk phytoestrogen contents associated with these diets was in the equol concentration; R2 and R3 yielded approximately 10-fold higher concentrations than B2 in Experiment 3 and S3 approximately eight-fold higher concentrations than L3 in Experiment 4. In both experiments, there was substantial variation among cows on the same diet in milk equol concentration.

For the lignans, no significant between-diet differences in milk concentrations of secoisolariciresinol or matairesinol were observed in Experiment 3, but B2 yielded higher concentrations of enterodiol and enterolactone than R2. In Experiment 4, there were no differences in the milk concentrations of secoisolariciresinol and matairesinol, but concentrations of the mammalian lignans enterodiol and enterolactone were higher in milk from cows on L3 than on S3.

The concentration of coumestrol was below the detection limit in all three silages and most milk samples analysed in Experiment 3. In Experiment 4, the concentration of coumestrol was higher in silage mixture L3 than in S3, but for both silages the concentration and intake were low. Its milk concentration was twice as high on L3 as on S3.

4.4 Paper IV

In the studies in Paper IV, the proportions in the milk and recoveries of C18:2n-6 and C18:3n-3 were higher on the red clover diets R2 and S3 than on B2 and L3, respectively, and the n-6/n-3 fatty acid ratio was higher on the red clover diets. Shortening the regrowth interval increased both the concentration of fatty acids in silage and the proportion of unsaturated fatty acids in milk.

For the vitamins, intakes of α-tocopherol, β-carotene and lutein were higher on B2 than on R2 due to the higher silage intake on B2. However, the highest vitamin intake was observed on diet R3 due to higher concentrations in the silage mixture. In Experiment 4, intakes of α-tocopherol, β-carotene and lutein were higher on L3. However, not all of these differences affected the milk, as the only significant differences related to silage diet in the two experiments were a higher milk α-tocopherol concentration on L3 than on S3 and a slightly lower β-carotene concentration on B2 than on R2. Supplementation with α-tocopherol increased milk α-tocopherol concentrations from 0.77 to 1.05 and from 1.07 to 1.24 mg/kg milk for S3 and L3, respectively, but did not affect milk fatty acid composition.

5 Discussion

5.1 Factors affecting concentrations of investigated compounds in plants

5.1.1 Effects of species

The choice of forage species affects both the phytoestrogen and fatty acid composition and concentration of the resulting feed, as reported in Papers I and II. There seems to be differences in these respects between the grasses and the legumes as groups, as well as between the species within each group.

The phytoestrogen analyses focused mainly on legumes, and the comparisons of both red clover with birdsfoot trefoil (Paper I) and red clover-grass silage with birdsfoot trefoil-grass or white clover-grass silage (Paper III) clearly showed that red clover has substantially higher concentrations of isoflavones than the other tested species.

In the fatty acid contents there were differences between meadow fescue and timothy, which seemed to be affected by factors such as site, year and cutting time. However, there were some interactions between species and cutting time, leading to larger differences between the species in the early cuts than in the later cuts. There were also higher leaf blade proportions in meadow fescue at Skara in 2005, than in the other trials, possibly due to low proportions of meadow fescue in the plots. This increased the total fatty acid concentration which was higher in meadow fescue than in timothy at this trial.

5.1.2 Effects of harvest time

Both the fatty acid and the phytoestrogen concentrations of forage are affected by the harvest time, as shown in Papers I and II. This is consistent with expectations because if the plants are harvested later they have a longer time to mature, and proportions of stems, leaves and inflorescence also change. Furthermore, as the plants advance from vegetative to reproductive stages, they develop more stems, supporting reproductive parts (spikelets or flowers), hence the leaf blade proportion falls. These trends have been previously observed in timothy by Gustavsson and Martinsson (2001) and Virkajärvi *et al.* (2012). In addition, Virkajärvi *et al.* (2012) found that the leaf to weight ratio differed between tillers in vegetative, generative stage and elongating vegetative stages. When the shoots enter reproductive stages the energy content of crops also decreases as the NDF concentration increases and digestibility of the DM and organic matter decline (Gustavsson & Martinsson, 2001; Nordheim-Viken *et al.*, 2009).

In the material examined in Paper I, the concentrations of formononetin and biochanin A decreased with later cutting time, in accordance with findings by Sarelli *et al.* (2003) that concentrations of isoflavones, mainly formononetin, in red clover decline with later cutting time. In contrast, Tsao *et al.* (2006) found higher total isoflavone concentrations in 13 green-house grown cultivars of red clover a late maturity stage (late flowering) than in an earlier (early bud stage) stage. In the summer cuts, the concentration of isoflavones in red clover seemed to be more strongly correlated to the stage of development than to the leaf blade proportions. Accordingly, the concentration of formononetin, primarily in red clover stems, but also in leaves and inflorescence, has been reported to decrease as the shoots mature (Sivesind & Seguin, 2005).

Similar to the findings presented in Paper II, an effect of advancing maturity on the fatty acid concentration has been previously recorded in timothy (Boufaïed *et al.*, 2003), in red clover and mixtures of timothy and meadow fescue (Vanhatalo *et al.*, 2007), and in perennial ryegrass, tall fescue and orchard grass (Mir *et al.*, 2006). The fatty acid concentration of the forage species seemed to be correlated with both the developmental stage and leaf blade proportion. The correlations between leaf blade proportion and fatty acid concentrations were slightly stronger for the grasses than for red clover, but this might have been due to minor variations in the development of red clover during the experimental period, as they were measured during the spring growth when red clover is usually at a vegetative stage. These effects of maturity stage could also be seen in the silage in Paper VI, with slightly higher concentrations of fatty acids and higher concentrations of α-tocopherol, β-carotene and lutein in silage from 3-cut red clover-grass silage than from 2-cut silage.

5.1.3 Effects of site

In Paper I, the concentrations of several isoflavones were higher at Umeå than at Skara. These differences between sites are confounded with an effect of cultivar as there were different red clover cultivars at the two sites. Other studies have recorded differences in the isoflavone concentrations between cultivars of red clover (Sivesind & Seguin, 2005; Saviranta *et al.*, 2008) and of the apigenin concentration in lucerne (Seguin & Zheng, 2006). Any comparison of the isoflavone concentration of the red clover cultivars Betty and Sara is, to the author's knowledge, not published.

Another possible explanation for the site difference is the higher leaf blade proportions at Umeå 2005 in the spring growth and the correlation between leaf blade proportions and isoflavone concentrations. Several studies have reported higher total concentrations of isoflavones in leaves than in stems (Sivesind & Seguin, 2005; Tsao *et al.*, 2006; Saviranta *et al.*, 2008). However, in the early stages of maturity the concentrations of biochanin A has been reported to be as high in stems as in leaves (Sivesind & Seguin, 2005).

Several factors differ between two the sites, for example photoperiod, temperature, soil moisture and type, notably Umeå is further north than Skara, hence the photoperiod is longer at Umeå during the summer. Effects of site on formononetin, biochanin A and total phytoestrogen concentrations in red clover have also been detected in an experiment in Canada (Sivesind & Seguin, 2005). Similarly, higher concentration of daidzein, genistein and glycitein have been detected in soybean cultivated at four sites in southern Brazil than at four sites in northern parts (Zhou *et al.*, 2011) and site effects have also been detected in soybeans grown in eastern Canada (Seguin *et al.*, 2004b). In addition, effects of temperature on isoflavone concentrations in red clover have been reported, including increased concentrations of formononetin in leaves of red clover grown under a 17°C/13°C, day and night temperature regime, than under higher temperatures (23°C/15°C) (McMurray *et al.*, 1986). Effects of light and temperature have also been seen in contents of other flavonoids, including anthocyanins, for example, higher anthocyanin concentrations in bilberries under light conditions similar to those of high latitudes than in others grown under light regimes typical of lower latitudes (Åkerström, 2010), and combined effects of light and temperature on flavonoids in several species, as reviewed by Jaakola and Hohtola (2010).

Also for the fatty acids, effects of site were seen (Paper II). When comparing the trials in Paper II, the botanical composition of the plots can explain some of the differences between the sites; the proportion of meadow fescue in the plots Skara 2005 was much lower than at Umeå 2005 and more shoots than normal had stayed in the leaf development stage for meadow fescue at Skara. This might explain the higher concentration of total fatty acids in meadow fescue at Skara 2005 than at Umeå 2005.

Environmental factors, such as solar radiation, temperature and precipitation might affect the fatty acid concentration. These environmental factors might affect the maturity stages in the crop. During spring growth, there seemed to be only small differences in the solar radiation between Umeå and Skara. Lower solar radiation has been correlated to higher total fatty acid concentrations in perennial ryegrass, but the results were confounded with changes in temperature (Witkowska *et al.*, 2008). Shading the crop prior to harvest have shown to decrease the fatty acid concentration, especially of C18:3 (Dewhurst & King, 1998).

As in Paper I, differences between sites in red clover might not be an effect of site; it might be an effect of variety as there were different clover varieties at the two sites. In a study by Van Ranst *et al.* (2009b) there was a tendency for differences in the fatty acid concentrations between different cultivars of red clover.

A previous study on α-tocopherol and β-carotene concentrations, in samples examined in studies appended to this thesis, also found effects of site, as plants grown at the southern site (Skara) generally had higher concentrations than plants grown at the northern site (Umeå) (Lindqvist, 2012).

5.1.4 Effects of year

In the cuts of the summer growth examined in Paper I there were minor differences in the isoflavone concentrations in red clover between years at Umeå, with slightly higher concentrations in 2005. In these cuts the red clover was slightly more mature in 2005 than in 2006, but the leaf blade proportion did not differ between the years.

In contrast, in the spring cuts, the fatty acid concentrations were generally higher at Umeå in 2006 than at Umeå in 2005 (Paper II). This can be explained, to some extent, by differences in cutting time, and hence developmental stages of the harvested shoots, since the harvests were earlier in 2006, thus the material would be expected to have higher fatty acid concentrations. The shoots at Skara in 2005 and 2007 were in a more similar developmental stage and (hence) there were smaller differences between the years.

However, the between-years differences at Umeå might also partly reflect differences between first year- and second year-leys or climate factors, particularly since daily mean temperatures were slightly higher during the beginning of the growth season, on average, at Umeå in 2006 than in 2005. In addition, there were slightly higher daily mean temperatures and slightly higher solar radiation during the early growth season at Skara in 2007 than in 2005.

5.1.5 Effects of wilting and forage conservation

The fields sampled in Experiment 2 in Paper I were harvested, and the crops were ensiled then used as feed in Experiment 3 in Papers III and IV. In the feeding experiment, the silage was analysed for phytoestrogen concentration, but as it was in mixtures with grass the phytoestrogen concentrations of the material used in the two studied cannot be compared in detail. However, several studies have compared concentrations in fresh and preserved material. One found higher concentrations of isoflavones in fresh red clover than in silage (Sivesind & Seguin, 2005), but another found the opposite (Sarelli *et al.*, 2003). In birdsfoot trefoil, the concentration of isoflavones reportedly decrease below the detection limit when ensiled (Sarelli *et al.*, 2003). This did not seem to occur in the material sampled in Experiment 3 in Paper III, as isoflavones were found in the birdsfoot trefoil-timothy silage and grasses apparently contain very low concentrations of isoflavones (Kallela *et al.*, 1987; Mustonen *et al.*, 2009). The concentration of isoflavones in red clover has also been reported to be higher in fresh material than in hay (Sivesind & Seguin, 2005). This might be due to losses of leaves during hay-making, as leaves often have high concentrations of isoflavones.

In terms of fatty acid concentration, no major differences were found between the fresh herbage analysed in Experiment 1 in Paper II and the silage used in Experiment 3 Paper IV. This is in agreement with findings that ensiling has little effect on fatty acid concentrations and composition (Dewhurst & King, 1998; Boufaïed *et al.*, 2003; Arvidsson *et al.*, 2009a).

5.1.6 Other sources of variation

In the fields, there were spatial variations within plots, although the plots were rather small and treated in the same manner. However, within a plot the maturity stage of the herbage, and its botanical composition, may vary. Both these factors may affect leaf blade proportions as well as the concentrations and composition of fatty acids and phytoestrogens.

Results presented in Papers I and II confirm that the botanical composition of swards may affect concentrations of these substances. For instance, a low proportion of one species in a mixture might affect the development of its shoots, as shown in Experiment 1 in Paper II, as the meadow fescue at Skara in 2005 was still in the leaf development stage at the cutting times and there were large proportions of leaf blades, possibly due to large proportions of red clover in the plots. Another, practical difficulty associated with small proportions of a species in plots is that it might be difficult to collect enough forage to obtain representative samples.

In all samplings it is important to ensure that the samples are collected in the same manner, at representative spots in the plots, silos or feed bunks. It is also important to make sure that any sub-samples analysed are representative of the samples they are drawn from. This is especially important in cases where the samples of a given species are small, as shown in Paper II. It is worth noting that a study of the effects of various methods of handling samples prior to fatty acid analysis found no any major differences in results between drying in an air-forced oven, freezing at -20°C or freezing with liquid N (Arvidsson *et al.*, 2009b).

5.1.7 Correlations

The concentrations of isoflavones and fatty acids seemed highly affected by the stage of maturity, as seen in Paper I and II. Changes in the leaf blade proportion can be explained by the stage of maturity. Therefore were there correlations between the leaf blade proportions and the concentrations of phytoestrogens and fatty acids. As the crop develops from the vegetative stage into reproductive stage the crop develops more stems to support the reproductive parts (spikelet or flowers). This leads to a decreased proportion of leaf blades. This has been reported in timothy by Gustavsson and Martinsson (2001) and Virkajärvi *et al.* (2012). This indicates that the leaf blade proportion is a good measure of the stage of maturity.

In Paper II, correlations between fatty acids and α-tocopherol and β-carotene were found, mainly in the grasses. Both the fatty acids and the β-carotene are located in the chloroplast and are found in high concentrations in the leaves. In the plants, β-carotene and α-tocopherol is quenching singlet oxygen and thereby protecting against photo oxidative stress, but they act at different locations (Asensi-Fabado & Munné-Bosch, 2010). The β-carotenes counteracts the chlorophyll-photosensitized formation of singlet oxygen whereas α-tocopherol protects the membranes (Asensi-Fabado & Munné-Bosch, 2010).

5.2 Factors affecting concentrations of phytoestrogens and fatty acids in milk

5.2.1 Effects of species

The differences in compositions and concentrations of phytoestrogens and fatty acids between birdsfoot trefoil and red clover, and between white clover and red clover, also affect both fatty acids and phytoestrogens in the milk from cows fed silage made from these forage species.

In Experiment 3 and 4, there were higher concentrations of all the analysed isoflavones in milk from cows on red clover-grass silage diets than in milk from cows on birdsfoot trefoil- or white clover-grass silage diets (Paper III). This appeared to be largely due to the differences in isoflavone concentrations between the legume species, as concluded in Paper I and other studies (Steinshamn *et al.*, 2008; Andersen *et al.*, 2009a). However, the silage DM intake is also an important factor. The high formononetin concentration in the summer cuts of red clover harvested in Experiment 2 of Paper I had major effects on the milk equol concentrations in feeding Experiment 3, as formononetin is the precursor of equol. The equol concentration in milk in Experiment 3 was higher than in previous published studies (Steinshamn *et al.*, 2008; Andersen *et al.*, 2009a; Mustonen *et al.*, 2009).

In marked contrast to the intake of isoflavones, intake of lignans was higher for cows on birdsfoot trefoil- (B2) and white clover-grass (L3) silage than on the red clover-grass silages (R2 and S3), and there were corresponding differences in milk lignan concentrations. The effects of birdsfoot trefoil silage on milk phytoestrogen concentrations has, to the authors knowledge not been published previously. The lignans found in the birdsfoot trefoil-timothy silage may have originated from both birdsfoot trefoil and timothy as the lignan concentration in timothy was not investigated and has, to the author's knowledge, not been published earlier. However, all lignans in the red clover-grass silages presumably originated from the grasses as no lignans were found in pure red clover (Paper I). However, some lignans may possibly have originated from the concentrate as there are other lignans than those analysed that are precursors of the mammalian lignans found in the milk (Heinonen *et al.*, 2001; Smeds *et al.*, 2007; Peterson *et al.*, 2010).

Concentrations of fatty acids differ little among the silages but, due to biohydrogenation, there were differences in the milk resulting milk, with higher proportions of C18:2n-6 and C18:3n-3 (and others) in milk from cows on the red clover diets R2 and S3 than in milk from cows on the birdsfoot trefoil diet B2 or white clover diet L3. This is consistent with expectations, since several previous studies have shown that rates of escape of polyunsaturated fatty acids from the rumen, and proportions of these fatty acids in the milk, are higher when red clover silage is ingested than when grass silages are ingested (Dewhurst *et al.*, 2003a; Vanhatalo *et al.*, 2007; Arvidsson *et al.*, 2012). This might be due to rumen passage rate of red clover being higher than that of grass (Kuoppala *et al.*, 2009), reduced lipolysis due to PPO (Lee *et al.*, 2004; Lee *et al.*, 2007; Lee *et al.*, 2009) or a combination of several factors. White clover diets also reportedly have a higher passage rate through the rumen than grass diets, and thus lower biohydrogenation rates and higher recovery of feed C18:2n-6 and C18:3n-3 (Dewhurst *et al.*, 2003a).

Birdsfoot trefoil contains condensed tannins (Dewhurst *et al.*, 2006), which have been shown to reduce rumen biohydrogenation *in vitro*, due to inhibition of the second biohydrogenation step (Jayanegara *et al.*, 2011). However, no such reduction was seen Experiment 3 in Paper III, as the proportion of *trans*-18:1 in milk was low.

5.2.2 Effects of harvest system

Increasing the regrowth interval of red clover-grass silage decreased both isoflavone and C18:2n-6 and C18:3n-3 milk concentrations (Papers III and VI). This was probably because the plants in the silage mixture from the three-cut system, R3, were less mature than those from the two-cut system, R2. Prolonging regrowth decreases the leaf blade proportion in grasses and legumes (Gustavsson & Martinsson, 2001; Vanhatalo *et al.*, 2007), and total fatty acid and C18:3n-3 concentrations in the plants (Dewhurst al., 2001), as most of the fatty acids are localized in the chloroplasts of the leaves. However, there were no differences between the diets in calculated apparent recoveries of C18:2n-6 or C18:3n-3 from feed to milk, indicating that the difference in the milk C18:3n-3 proportion between cows fed R2 and R3 was mainly due to a higher intake of the preferred fatty acid on R3 rather than differences in the biohydrogenation of the fatty acids.

5.2.3 Effects of supplementation of α-tocopherol

In Experiment 4 in Paper IV, there were higher concentrations of α-tocopherol in milk from cows supplemented with α-tocopherol than the in milk on unsupplemented diets. This is in agreement with several previous studies (Al-Mabruk *et al.*, 2004; Meglia *et al.*, 2006; Lindqvist *et al.*, 2011).

5.2.4 Variations between cows

In the studies in Paper III, variations among animals in the concentration of equol in milk were found, despite similar phytoestrogen (formononetin and daidzein) intakes. Some of the variations could be due to differences in feeding rates. Lundh *et al.* (1990) also recorded between-animal differences in plasma concentrations of phytoestrogens, and found that formononetin and daidzein (both free and conjugated) were absorbed rapidly after feeding, reaching maximal plasma concentrations within an hour, but the absorption of equol was not affected by the feeding time.

Differences in equol-related physiology between human individuals have also been found, as reviewed by Atkinson *et al.* (2005). Indeed, in contrast to cows, humans can be divided into good and poor equol excretors according to (Rowland *et al.*, 2000), who found that 36% of participants eating a high-isoflavone diet were good equol excretors, and had high levels of equol in their urine. Further, the good excretors consumed less dietary fat and more carbohydrates as a percentage of their total energy intakes than the poor excretors. The cited authors proposed that high fat intake might decrease the capacity of gut microbial flora to synthesize equol. Other studies have also found correlations between low fat-high fibre diets and high production of equol and mammalian lignans (Lampe *et al.*, 1999). However, another study found positive correlations between fat and meat intakes and equol concentration in urine (Adlercreutz *et al.*, 1991).

Comparing these findings with those presented in Paper III, the variations found between cows were probably not due to variations in fat intake but other factors, as all the cows in the trial group were fed the same diets and the differences in milk were not correlated with the minor within-diet that were found. Furthermore, cows included in Experiment 3 had higher fatty acid intakes than those in Experiment 4 (Paper IV), but higher milk equol concentrations (Paper III). However, as the cows were kept in different environments, other factors such as their rumen microbial flora probably differed substantially.

5.2.5 Effects of phytoestrogens on cows

In the experiments this thesis is based upon the effects of the treatments on animal health were not investigated. However, no cows were withdrawn from the experiments due to mastitis or other detected health problems, and no negative effects on fertility were reported from the research stations after the experiments. This is not surprising, partly because cows seem to be less affected by phytoestrogens than ewes (Adams, 1995) and partly because in the applied change-over design of the experiments the period when the cows were fed silage high in isoflavones was short (at most six weeks, for cows sequentially fed both red clover silage diets), and probably too short to cause any negative effects, on fertility for example. The higher concentrations of α-tocopherol and β-carotene found in some of the silages might be beneficial for the animals. However, they did not affect milk concentrations, in accordance with a previous report of correlations between high intake of α-tocopherol and increased plasma α-tocopherol concentrations, with no accompanying increase in milk concentrations (Lindqvist *et al.*, 2011).

5.2.6 Intake of phytoestrogens and fatty acids, and effects on human consumers

The mean human intake of whole milk is reported to be 351 and 255 g/day in the U.S. and Europe, respectively (FAOSTAT, 2010). If milk from cows fed diet R2 was consumed at those rates, it would result in mean equol intakes of 520 and 380 µg equol/day for the U.S. and European populations, respectively. If milk from cows fed diet L3 was consumed instead, the resulting intake of equol would be about 25 µg equol/day. In comparison, total intakes of between 1,200 and 1,800 µg/day of phytoestrogens (Cotterchio *et al.*, 2008; Ward *et al.*, 2010), 1,000 µg/day of isoflavones (Chun *et al.*, 2009) and 360 µg/day of daidzein (Frankenfeld, 2011) have been reported. However, most people have substantially lower phytoestrogens intakes, because the means are biased by small proportions of people who consume relatively large amounts of soybeans and soy-containing foods (Ward & Kuhnle, 2010; Frankenfeld, 2011).

In humans, not all individuals have the ability to produce equol from plant precursors, as stated earlier (Rowland *et al.*, 2000; Atkinson *et al.*, 2005), and the beneficial health effects of isoflavones are thought to depend on this ability (Atkinson *et al.*, 2005; Jackson *et al.*, 2011). Indeed, any potential benefits of consuming equol precursors will be lower for people who do not have the ability to produce equol than for people that have the ability, or even non-existent. For people who do have the ability, phytoestrogen metabolites in milk might make contributions to the beneficial effects of phytoestrogens. Accordingly, in intervention health studies plasma equol concentrations have been found to be higher in individuals who showed health improvements (reductions in hot flashes, increased bone mineral density and decreased LDL-cholesterol levels), as reviewed by Jackson *et al.* (2011).

In similar comparisons of fatty acid intakes, the mean intakes of C18:3n-3 acid resulting from drinking milk from cows fed diets B2, R2, R3, L3 and S3 would be 0.38, 0.50, 0.65, 0.6 and 0.8 g/day, respectively, in the U.S., and 0.28, 0.37, 0.40, 0.43 and 0.58 g/day, respectively, in Europe. The corresponding intakes of C16:0 would be 23.2, 23.6, 23.0, 32.3 and 32.9 g/day in the U.S. and 16.8, 17.2, 16.7, 23.4 and 23.9 g/day in Europe. These calculations indicate that the effects of varying cows' diets on milk fatty acid concentrations are smaller than the differences between trials and differences in milk consumption between regions.

To conclude this brief review of health effects of the investigated substances, Bonthuis *et al.* (2010) found that subjects with the highest intakes of full-fat dairy products had lower rates of mortality due to cardio vascular disease than subjects with the lowest intakes.

5.2.7 Choice of phytoestrogens to analyse

Other phytoestrogens are present in forage, concentrate and milk, in addition to those analysed in the studies this thesis is based upon. However, the most important isoflavone for animal and human health are formononetin and daidzein, due to the associated equol production. Genistein is also important due to its high affinity for oestrogen receptors (Pfitscher *et al.*, 2008) and its high concentrations in soybean and soy-products (Cederroth & Nef, 2009). Coumestrol also has high binding affinity for the receptors, but was only detected in some samples and at low concentrations. For the lignans, other precursors of the mammalian lignans could have been analysed, because cereals (for example) contain other lignans that are precursors of the mammalian lignans enterodiol and enterolactone (Heinonen *et al.*, 2001; Smeds *et al.*, 2007). In experiments with even higher concentrate rations, this would be of greater importance. When studying phytoestrogens, it is also important to remember their difference in binding affinities for oestrogen receptors (Pfitscher *et al.*, 2008). The phytoestrogen present in the highest concentrations is not necessarily the most interesting.

6 Conclusions

The main conclusion from the presented studies is that several factors affect concentrations of phytoestrogens, fatty acids and vitamins in forage, silage made from forage and milk from cows fed silage, including the following.

There are substantial variations between species in concentrations of phytoestrogens and fatty acids; red clover was found to have higher concentrations of isoflavones and birdsfoot trefoil higher concentrations of lignans. Meadow fescue had higher concentrations of most fatty acids than timothy, but there were less clear differences in their concentrations between red clover and birdsfoot trefoil.

Delaying harvest in spring growth reduced the concentrations of most phytoestrogens and, by slightly different mechanisms, fatty acids. The phytoestrogen concentrations seemed to be most closely linked to the developmental stage, whereas the fatty acid concentrations were associated with both the developmental stages and the leaf blade proportion in grasses. Prolonging the regrowth interval decreased phytoestrogen concentrations in pure, fresh red clover, and decreased fatty acid, α-tocopherol, β-carotene and lutein concentrations in silage. Apparently, this was mainly due to increased maturity of the plants. The harvest time in spring growth had minor effects on isoflavone concentrations in red clover during the summer.

Site effects on both isoflavone and fatty acid concentrations were detected; concentrations of isoflavones generally being higher, but fatty acid concentrations lower, at Umeå than at Skara. However, for red clover, the effect of site was confounded with the effect of variety. Some of these differences could be explained by differences in developmental stage of the herbage at the sites but there seems to be other unidentified factors as well.

Feeding cows red clover-grass silages increased isoflavone concentrations and proportions of C18:2n-6 and C18:3n-3 in milk, but decreased milk lignan concentrations compared to feeding birdsfoot trefoil- or white clover-grass silages. The milk concentration of equol was high on the red clover-grass diets at Umeå, and there were large variations between cows at both sites despite similar intake. No negative effects of fertility of the cows were observed. There were higher milk isoflavone concentrations and milk proportions of C18:2n-6 and C18:3n-3 when cows were fed three-cut rather than two-cut red clover-grass silage. Milk α-tocopherol concentrations increased with supplementation and there were higher concentrations in milk from cows fed white clover-grass silage than in milk from cows fed red clover-grass silage.

7 Future perspectives

Before increasing the use of legumes, such as red clover, in sustainable production systems, it is important to characterise their phytoestrogen contents thoroughly, and ensure that they will have no negative effects. To achieve these aims, further studies are required to identify factors responsible for between-site and between-year differences (*inter alia*) in isoflavone concentrations in the plants, and the strength of their effects. The effects of phytoestrogens on cattle also require further study (although no clear evidence of negative health effects of isoflavones on dairy cows, such as fertility problems, has been published to date). This is because if red clover-derived feeds (or others) were used to develop phytoestrogen-rich milk, it is clearly imperative to ensure that neither the cows nor their calves are negatively affected. Further studies on the effects of phytoestrogens in milk on human consumers are also needed, if aiming to increase milk phytoestrogen concentrations.

The variation between animals in milk equol concentrations also needs to be further investigated, to determine whether the observed variations are generally substantial, or were specific to the reported experiments. In addition, in either case the underlying reasons for the differences need to be identified. In humans, some factors affecting equol-related physiology seem to have been found, and it is possible that the interindividual differences in cows are caused by similar factors.

Some of the factors affecting concentrations of fatty acids in plants have been identified, such as species, harvest time, site and wilting. Various studies have also elucidated aspects of their metabolic pathways in the rumen. However, most unsaturated fatty acids in forage are not transferred to milk, raising questions about the suitability of using forage to modulate milk fatty acid concentrations in order to increase intakes of beneficial fatty acids, rather than perhaps focusing on other possible ways.

8 Populärvetenskaplig sammanfattning

Mjölk har inte ett konstant näringsinnehåll utan dess sammansättning kan påverkas av olika faktorer som till exempel utfodring, avel samt skötsel av djuren. Vissa av de ämnen som finns i mjölken kan ha hälsoeffekter på människor.

I denna avhandling undersöks två av dessa grupper med potentiell hälsoeffekt, så kallade fytoöstrogener och fettsyror. Syftet är att undersöka hur halten av fytoöstrogener och fettsyror varierar i olika vallväxter beroende på skördetid och växtplats och hur detta påverkar mjölkens sammansättning när dessa vallväxter utfodras till mjölkkor. Mjölkens innehåll av vitaminer studeras också samt hur ett tillskott av vitamin E till korna påverkade mjölkens vitamininnehåll. Dessutom undersöktes sambandet mellan halten av fettsyror i vallväxterna och deras vitamin och protein innehåll.

Fytoöstrogener är en grupp av ämnen som finns i vallväxter och i andra växter. Det är ämnen i växter som liknar de könshormoner som finns hos däggdjur (och hos människor). Denna likhet gör att de kan fungera som ersättning för östrogener och blockera eller starta en hormonreaktion, de kan alltså både ha en östrogen och en antiöstrogen effekt. Tidigare studier har visat att får som utfodras med fytoöstrogener kan få stora problem med fruktsamheten men hos nötkreatur har inte några tydliga tecken på problem konstaterats. Även människor kan eventuellt påverkas av fytoöstrogener, främst har det rapporterats minskad förekomst av hormonberoende cancerformer som bröst- och prostatacancer hos människor som äter stora mängder mat t ex sojabönor innehållande fytoöstrogener.

Fytoöstrogener finns i baljväxter såsom soja, klöver och lusern och i fiberrika växter såsom råg och vallgräs. När mjölkkor utfodras med foder innehållande fytoöstrogener bryts en del ner i vommen, andra passerar vommen oförändrade, medan andra ombildas till nya ämnen med större östrogen effekt. En liten del av dessa utsöndras i mjölken men mycket utsöndras med urinen.

Vad gäller mjölkens fettsyresammansättning är en stor andel av fettet mättat. Mättat fett har länge ansetts vara en risk för utveckling av hjärt-kärlsjukdomar och andra hälsoproblem. Mjölkfett innehåller även fettsyror som kanske har positiva hälsoeffekter. Till exempel finns en särskild sorts omättade fettsyror kallade konjugerad α-linolensyra (CLA). Dessa fettsyror har i provrörsförsök visat sig ha flera positiva hälsoeffekter, t ex minskad tillväxt av cancerceller. Mjölkfett innehåller också låga halter av de fleromättade fettsyrorna linolsyra och α-linolensyra. Dessa två fettsyror är essentiella för alla däggdjur, det vill säga dessa behöver tillföras med kosten och kan inte produceras i kroppen.

Mjölkens fettsammansättning kan påverkas genom utfodringen. Fettet som finns i vallfoder innehåller en stor andel omättade fettsyror, särkilt α-linolensyra och linolsyra. När kor utfodras med omättade fetter kommer mikroorganismer i kons första mage, vommen, att göra om de flesta av dessa fettsyror till mättade fetter. Men en del fettsyror kan passera vommen för att sedan tas upp i kons kropp.

Olika vallväxter har olika hög andel fettsyror och olika sammansättnig på fettsyrorna. När rödklöver utfodras kommer en större andel av de omättade fettsyrorna linolsyra och linolensyra undvika nedbrytning i vommen än när t ex majs eller en stor andel kraftfoder utfodras. En nackdel med en ökad mängd omättade fettsyror i mjölken är att dess hållbarhet kan minska på grund av att fettet lättare härsknar (fettsyrornas dubbelbindningar reagerar med syre). På grund av detta vill man gärna höja koncentrationen av skyddande antioxidanter, t ex fettlösliga vitaminer så som vitamin E (tokoferol) och provitaminer så som karotenoider, som hindrar fettsyrorna från att oxidera.

In den här avhandlingen undersöktes innehållet av växternas fytoöstrogen- och fettinnehåll i ett fältförsök på två orter (Umeå och Skara) under två säsonger på varje ort. Fytoöstrogeninnehållet undersöktes i rödklöver och dessutom gjordes en jämförelse mellan rödklöver och käringtand. Fettsyrakoncentrationen undersöktes i fyra vallväxter i blandningar; timotej jämfördes med ängssvingel, båda i blandningar med rödklöver och rödklöver jämfördes med käringtand, båda i blandningar med timotej.

Resultatet visade att rödklöver innehåller höga halter av fytoöstrogener av gruppen isoflavoner. Vid senare skördedatum i första skörd, det vill säga vid ett senare utvecklingsstadium, sjönk halterna av de flesta fytoöstrogenerna. En längre period mellan första och andra skörd sänkte också halterna av de flesta fytoöstrogener, även det troligen på grund av mer utvecklade växter. Generellt var det högre halter av fytoöstrogener i rödklöver skördade i Umeå än i Skara.

Denna skillnad kan bero på skillnader mellan de sorter av rödklöver som användes, skillnader i hur utvecklad rödklövern var eller skillnader mellan ortsspesifika faktorer såsom solinstråling, temperatur, dagslängd med mera. När fytoöstrogeninnehållet i käringtand jämfördes med det i rödklöver visade alla fytoöstrogener av gruppen isoflavoner mycket lägre halter i käringtand än i rödklöver medans fytoöstrogener i gruppen lignaner bara kunde uppmätas i käringtand.

Vad gällde koncentrationen av fettsyror i vallväxterna visade resultaten på skillnader mellan arter vad gällde innehållet av fettsyror. Ängssvingel hade, för ett flertal olika fettsyror, en högre koncentration än vad det var i timotej. Rödklöver hade högre koncentration än käringtand av vissa fettsyror medans för andra var det tvärtom. Den totala halten av fettsyror skilde sig inte mellan baljväxterna. Precis som för fytoöstrogenerna påverkades halten av olika fettsyror i växterna av skördetiden. Tidigare skördade växter hade generellt en högre koncentration än när växterna skördades senare i första skörd. Detta berodde troligen främst på växternas utvecklingsstadium och på hur stor viktandel av växten som var blad i förhållande till övriga växtdelar. Blad innehåller högre koncentration fettsyror än vad stammar och blommor har. Det fanns även skillnader mellan orterna som växterna odlades på, koncentrationen av fettsyror var ofta högre i Skara än i Umeå. Detta kan till viss del förklaras av att växterna var i ett senare utvecklingsstadium i Umeå.

Mjölkens fytoöstrogen-, fett- och vitamininnehåll undersöktes vid två utfodringsförsök med mjölkkor (i Umeå och på Ås, Norge). I försöket i Umeå utfodrades tre olika ensilageblandningar; rödklöver, ängssvingel och timotej skördat tre gånger under växtsäsongen; rödklöver, ängssvingel och timotej skördade två gånger samt käringtand och timotej skördade två gånger. I försöket på Ås utfodrades ensilage från en långliggande vall dominerad av vitklöver och gräs samt ensilage från en kortliggande vall dominerad av rödklöver och gräs.

Resultaten från de två utfodringsförsöken visade att innehållet av fytoöstrogener och vitamin var högre i ensilaget om vallen skördades vid tre tillfällen under säsongen (med kortare tid mellan skördarna) än vid två tillfällen. Innehållet av fytoöstrogener av gruppen isoflavoner var högre och av gruppen lignaner var lägre i ensilage med rödklöver och gräs än med käringtand eller vitklöver och gräs. Vitamininnehållet var lite högre i vitklöver-gräsensilage än i rödklöver-gräsensilage på Ås. Fettsyreinnehållet skilde inte så mycket mellan artblandningarna i ensilagen.

I mjölken var det betydligt högre koncentrationer av isoflavoner när korna hade utfodrats med rödklöver-gräs ensilage jämfört med käringtand- eller vitklöver-gräs ensilage. Särskilt höga var koncentrationerna av fytoöstrogenen equol och dessa koncentrationer varierade stort mellan kor trots att de ätit lika mycket av förstadierna till denna substans. Koncentrationen av lignaner visade motsatt resultat, högst koncentrationer i mjölk var det när korna utfodrats med käringtand- eller vitklöver-gräs ensilage. Utfodringen orsakade inga negativa effekter på kornas fruktsamhet eller hälsa.

Koncentrationen av fettsyror i mjölken var högre av linol- och α-linolensyra när korna utfodrades med rödklöver-gräs ensilage än med käringtand- eller vitklöver-gräs ensilage. Vitaminhalten i mjölken påverkades inte av de olika utfodringarna. När korna utfodrades med rödklöver-gräs ensilage från treskördesystemet, var det högre koncentration av linol- och α-linolensyra än när de utfodrades med ensilage från tvåskördesystem. Detta trots att de ät lika mycket av dessa fettsyror. Detta kan förklaras med skillnader i nedbrytningen av dessa fettsyror i vommen. När korna fick ett fodertillskott med semi-naturligt vitamin E ökade halten i mjölken men det påverkade inte mjölkens fett- eller fytoöstrogeninnehåll.

Slutsatsen av dessa försök är att utvecklingsstadiet har stor effekt på koncentrationen av både fytoöstrogener och fettsyror i vallväxter och genom att skörda i ett tidigare stadium då växterna har en stor andel blad kan man öka koncentrationerna. Men det finns även skillnader mellan orter och odlingsår som kräver fler undersökningar för att kunna förklaras. I mjölken får man högst koncentration av fytoöstrogener (av gruppen isoflavoner) om man utfodrar rödklöver-gräs ensilage och man får högre proportion av linol- och α-linolensyra om man utfodrar ensilage från ett treskördesystem jämfört med ett tvåskördesystem. Det behövs fler studier som undersöker om höga halter av fytoöstrogener i mjölk kan påverka människors hälsa, såsom anticancerogena effekter. Det behövs också studier på effekten av olika fettsyror från mjölk på humanhälsan.

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