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Occurrence of phytoestrogens in rations of Austrian dairy cows

Diploma thesis

University of Veterinary Medicine Vienna

submitted by

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Abbreviations

AMS	automatic milking system
DM	dry matter
E_2	17β-estradiol
e.g.	exempli gratia (for example)
ER	estrogen receptor
i.e.	id est (that is)
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MAT	Matairesinol
O-DMA	O-desmethylangolensin
PMR	partial mixed ration
SECO	Secoisolariciresinal
TMR	total mixed ration
ZEN	Zearalenone

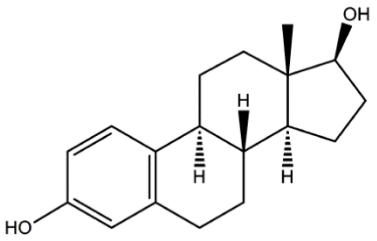
1. Introduction

In modern dairy farming, nutrition and feeding management play vital roles on farm workload and rentability. The diet strongly influences on health, reproductive and productive performance of dairy cows. Optimal fertility is a key factor to a farms well doing and one of the most decisive factors in a cows life. Phytoestrogens are secondary metabolites produced in some plants and forages for human and animal nutrition, which can have biological activity, agonizing or antagonizing endogenous estrogens. The main sources of phytoestrogens for cows are plant of the family Leguminosae plants. In high levels, those compounds can adversely affect the reproductive and productive performance of dairy herds, harming the efficiency and profit of the dairy farms. This study aimed to determine the occurrence and dietary levels of ten phytoestrogens in rations of lactating dairy cows from Austrian dairy farms. The hypothesis of this work is, that the TMR of organic farms contains a significant higher amount of phytoestrogens than the rations of conventional farms.

2. Literature review

2.1. Phytoestrogens

Phytoestrogens are plant compounds that are functionally or structurally similar to mammalian estrogens, especially 17β-estradiol (E2) (Mostrom and Evans 2011, Mostrom and Evans 2018). Its chemical structure is shown in Figure 1. They are secondary metabolites produced during photosynthesis and play important biological roles in plant growth, development and maintenance (Hashem and Soltan 2015). Studies showed that phytoestrogens have estrogen-like effects on multiple organ systems in animals (Adams 1995a, 1995b, Kallela et al. 1984). Not only the quantity of phytoestrogens and amount of metabolites but also the time of exposure has a big influence on their effect. Research on phytoestrogens and especially on their biological effects is still scarce (Burton and Wells 2002).



17BETA-ESTRADIOL

(8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthrene-3,17-diol

Figure 1. Chemical structure of 17β estradiol (Mostrom and Evans 2018)

Multiple literature sources discussing the effects of phytoestrogens in humans are available. Some studies refer to beneficial effects regarding diabetes, atherosclerosis, angiogenesis, osteoporosis, and hot flushes at menopause. Other investigations highlight their probiotic, antineoplastic, anti-inflammatory and antioxidant activity (Mostrom and Evans 2018). However, in high concentrations, phytoestrogens can also be viewed as endocrine disruptors in domestic animals like cows, meaning that they can impair health, production and reproduction (Reed 2016, Woclawek-Potocka et al. 2005, Woclawek-Potocka et al. 2008). Due to the rapidly increasing global consumption of phytoestrogens by processed foods and dietary supplements, which are primary sources of phytoestrogens, such as soy protein, clarity and additional research on this issue is needed (Patisaul and Jefferson 2010).

Publications concerning phytoestrogens in livestock mainly focus on their adverse effects on animal reproductive processes (Reed 2016, Woclawek-Potocka et al. 2005, Woclawek-Potocka et al. 2008). A recent review (Tarkowská 2019) provides evidence that plants can synthesize animal steroid hormones like E2, progesterone, testosterone, and others. They also conclude that while finding all classes of animal steroids in plants, it doesn't imply them all being hormonally active.

2.2. Chemical structure

As previously mentioned, phytoestrogens got their name from their chemical structure, which resembles E2. Due to the similar molecular structure, these plant compounds may bind with the same estrogenic receptors (ER) as endogenous estrogens/ hormones like E2 (Ososki and Kennelly 2003). The fundamental requirements for this receptor-ligand binding are the hydrophobic and steric properties of a compound and the hydrogen bonding between the ER binding site and the phenolic hydroxyl group of the phytoestrogens (Hu and Aizawa 2003). Phenolic compounds are aromatic benzene ring compounds with one or more hydroxyl groups (Hättenschwiler and Vitousek 2000).

More than 100 molecules of phytoestrogens are known. They can further be grouped according to their chemical structures (Hashem and Soltan 2015). The first big differentiation is between steroidal estrogens found in a few plants and the more commonly occurring nonsteroidal phytoestrogens. Examples of steroidal phytoestrogens are β-sitosterol, which is commonly found in most plants, and estrone from date palm (*Elaeis guineensis*) (Ososki and Kennelly 2003). Nonsteroidal phytoestrogens are polyphenols and include lignans, stilbenoids, tannins and several flavonoids without limitation. Flavonoids are classified into many types, e.g. isoflavones, flavones and coumestans (Adzersen and Strowitzki 2003, Benassayag et al. 2002, Mostrom and Evans 2011). Some mycotoxins such as zearalenone (ZEN) and alternariol possess estrogenic potential as well, but due to their fungal origin, these molecules are classified as mycoestrogens. These fungal molecules can interact synergically with the phytoestrogens (Grgic et al. 2021).

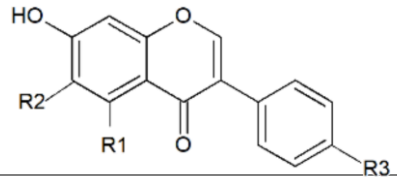
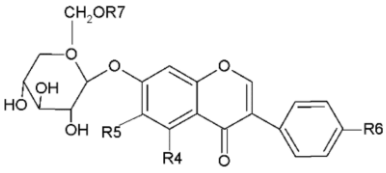
2.2.1. Isoflavones

Among all phytoestrogens isoflavones are the most well-known. They became widely renowned after discovering clover disease (Ososki and Kennelly 2003). It has been reported that Australian sheep mainly fed with subterranean clover (*Trifolium subterraneum*) suffered from reproductive issues. They showed reduced lambing rates, abnormal lactation, genital changes, infertility, dystocia and uterine prolapse (Bennetts et al. 1946).

The chemical structures of isoflavones and their glycosides are shown in Table 1. Ubiquitous occurring isoflavones that show estrogenic activity are the glycosides daidzin and genistin (Price and Fenwick 1985). Once the glycosyl group from the glycosides is replaced with a hydrogen atom, they turn into the aglycones daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone), respectively (Moss et al. 1995). These aglycones can be further

transformed into their 4'methyl ethers, for instance, biochanin A and formononetin (Kurzer and Xu 1997, Price and Fenwick 1985). In plants they are usually present as glycosides (Ibarreta et al. 2001). During processing, isolation and analysis they eagerly change enzymatically or chemically into aglycones (Price and Fenwick 1985). Another isoflavone with reported estrogenic activity is glycitein (4',7-dihydroxy-6-methoxyisoflavone) (Song et al. 1999).

Table 1. Chemical structures of isoflavones and their glycosides (Bingham et al. 1998, Daems et al. 2016, Křížová et al. 2019)

Aglycon		**R1**	**R2**	**R3**	
Daidzein		H	H	OH	
Genistein		OH	H	OH	
Glycitein		H	OCH_3	OH	
Formononetin		H	H	OCH_3	
Biochanin A		OH	H	OCH_3	
Glucoside		**R4**	**R5**	**R6**	**R7**
Daidzin		H	H	OH	H
Genistin		H	H	OH	H
Glycitin		H	OCH_3	OH	H
Ononin		H	H	OCH_3	H
Sissotrin		OH	H	OCH_3	H
Acetyldaidzin		H	H	OH	$COCH_3$
Acetylgenistin		OH	H	OH	$COCH_3$
Acetylglycitin		H	OCH_3	OH	$COCH_3$
Malonyldaidzin		H	H	OH	$COCH_2COOH$
Malonylgenistin		OH	H	OH	$COCH_2COOH$
Malonylglycitin		H	OCH_3	OH	$COCH_2COOH$
Malononin		H	H	OCH_3	$COCH_2COOH$
Malonylsissotrin		OH	H	OCH_3	$COCH_2COOH$

2.2.2. Stilbenoids

The most well-known stilbenoid is resveratrol (3,5,4'-trihydroxystilbene), a derivate of stilbene. There are two geometric isomers: *trans*- and *cis*-, (Figure 2) with the *trans*-isomer appearing more frequently. It can be found conjugated to glucose (Mattivi et al. 1995). When exposed to ultraviolet irradiation, the *trans*-from can undergo photoisomerization and change into the *cis*-form (Lamuela-Raventos et al. 1995).

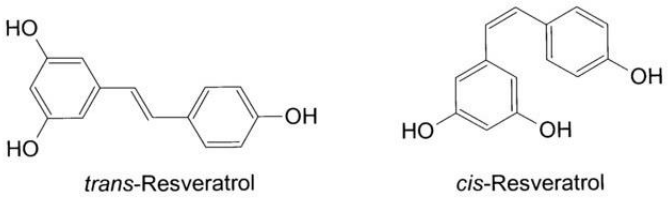


Figure 2. Chemical structures of trans- and cis-Resveratrol (Aziz et al. 2003)

2.2.3. Lignans

Lignans are a structurally diverse class of secondary metabolites (Figure 3), which are commonly detected in the plant kingdom. They are defined as dimeric phenylpropanoid (C_6-C_3) compounds and are mostly linked 8-8'. Nowadays, many other linkage types are included within the term lignan and share their primary purpose for plant defence (Lewis and Davin 1999). Lignans are stored in plant vacuoles as glycosides. In humans, they are converted into active phytoestrogens by gut microflora (Adlercreutz 2002). Thereby the glycosides of matairesinol (MAT) and secoisolariciresinol (SECO) are metabolized into enterolactone and enterodiol, the so-called "mammalian lignans". Amazingly enterodiol can be converted into enterolactone just like the precursors of SECO pinoresinol, lariciresinol and syringaresinol (Adlercreutz 2002). This shows that many ubiquitous occurring lignans can be converted into phytoestrogens (Dixon 2004).

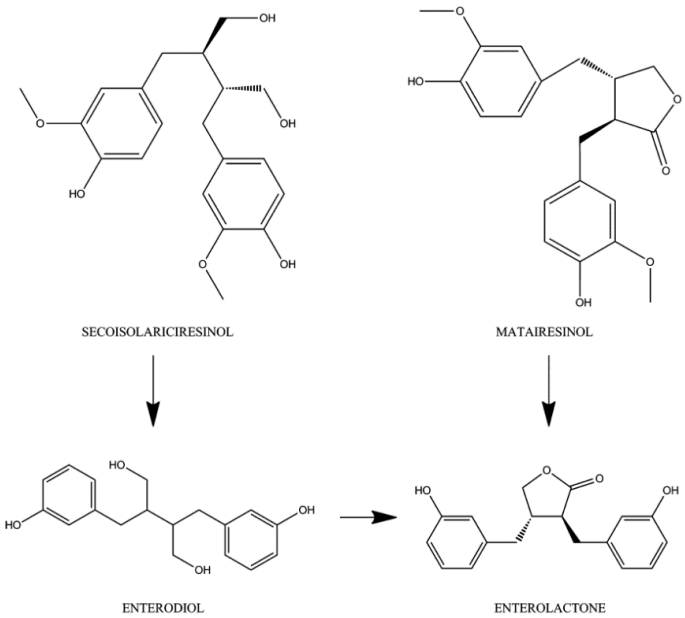


Figure 3. Chemical structures of plant and mammalian lignans (Mostrom and Evans 2018)

2.2.4. Flavones

Flavones are types of flavonoids with apigenin (4',5,7-trihydroxyflavone) and luteolin (3',4',5,7-Tetrahydroxyflavone) being the most relevant compounds of this group (Patisaul and Jefferson 2010). Their precursors are the aromatic amino acids L-tyrosin and L-phenylalanine (Herrmann 1995).

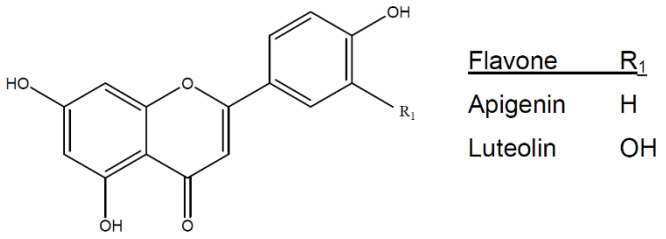
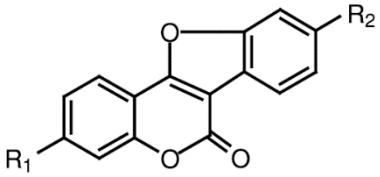


Figure 4. Chemical structure of flavones (Bhagwat and Haytowitz 2016)

2.2.5. Coumestans

A group of plant phenols that belong to flavonoids and show estrogenic activity are coumestans (Ososki and Kennelly 2003). They were first reported in 1957 when coumestrol was isolated from strawberry clover (*Trifolium fragiferum*), ladino clover (*Trifolium repens*) and lucerne (also called alfalfa) (*Medicago sativa*) (Bickoff et al. 1957). Until now at least 27 coumestans have been described (Reed 2016), with the main coumestans being coumestrol (7'12'-dihydroxy coumestan) and 4'-methoxycoumestrol (Figure 5) (Whitten and Naftolin 1998). Their primary purpose is to protect the plant from stress (Bhattacharya et al. 2010).



Coumestan	R_1	R_2
Coumestrol	OH	OH
4′-Methoxycoumestrol	OH	OCH_3

Figure 5. Chemical structures of coumestans (Ososki and Kennelly 2003)

2.2.6. Mycoestrogens

Some mycotoxins, toxic secondary metabolites produced by molds, have estrogenic effects as well. Macrocyclic resorcyclic acid lactone compounds, such as ZEN and its derivates (Figure 6), are the most prominent representatives.

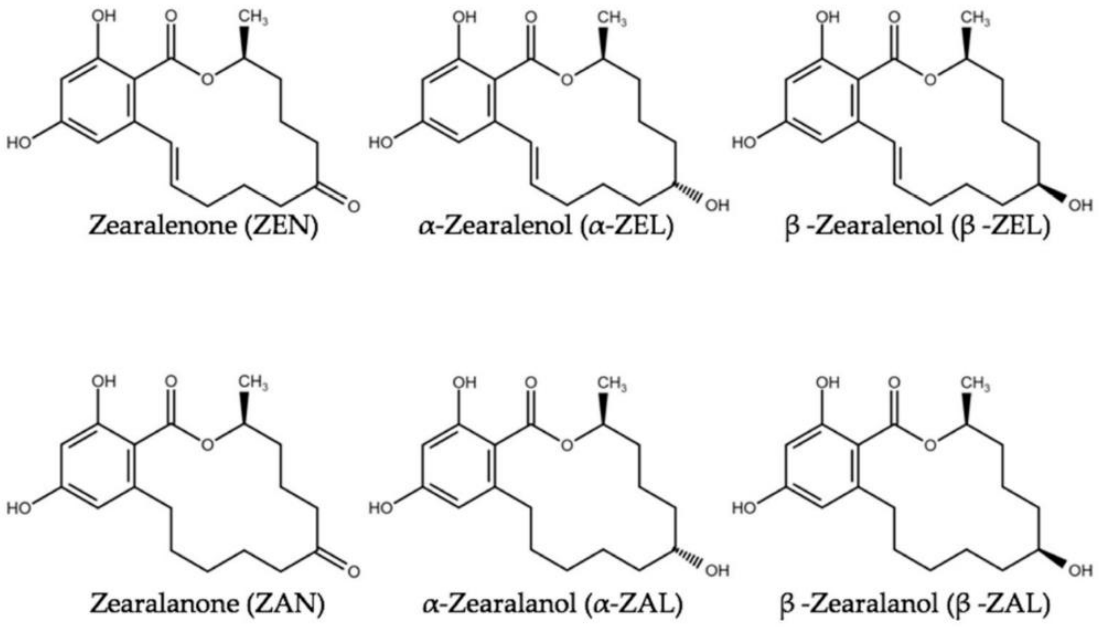


Figure 6. Chemical structure of the mycotoxin zearalenone and its phase I metabolites (Grgic et al. 2021)

ZEN is widely distributed and mainly produced by *Fusarium graminearum*, *F. crookwellense* and *F. culmorum.* It is usually associated with corn but can also appear in barley and wheat (IARC 1993). Other mycoestrogens are alternariol and alternariol methyl-ether, which are produced by *Alternaria* spp. (Vejdovszky et al. 2016). These fungal compounds can interact with the phytoestrogens, inducing additive/synergist effects on the estrogenic activity (Hessenberger et al. 2017, Montes-Grajales et al. 2018, Nikov et al. 2000, Salom et al. 2007). A recent survey study in Austria showed that both mycoestrogens and phytoestrogens co-occurred in the diets of dairy cows (Penagos-Tabares, Khiaosa-ard et al. 2022).

2.3. Main sources of phytoestrogens

The phytoestrogen concentrations differ among plant materials. The metabolism of phytoestrogens occurs in specific plant tissue, which also varies between phytoestrogenic compounds and plant species, and does not allow phytoestrogens to translocate via vascular pathways. For example, there are higher concentrations of coumestrol near the top (apical) part of the lucerne plant compared to the lower parts (Seguin et al. 2004). Table 2 shows quantitative concentrations of some phytoestrogens in legumes, oil seeds, nuts, cereal grains and processed products which were analysed using isotope dilution gas chromatography-mass spectrometry with selected ion monitoring (Mazur et al. 1998). Legumes are the richest source of phytoestrogens in animal diets. Particularly, soy beans and clovers often constitute a large part of the rations, therefore increasing the ingested doses and possibly their impacts (Hashem and Soltan 2015). Some reports state that commonly used forage legumes like subterranean clover (*Trifolium subterraneum*) and soybean (*Glycine* sp.) may contain estrogenic isoflavones, respectively, up to 5 and 0.25% of dry weight (Adams 1995a).

The predominant phytoestrogens in soybean are the isoflavones daidzein, genistein and glycetin. The phytoestrogens with the highest concentrations in clover are biochanin A and formononetin. The main phytoestrogens in flaxseeds are lignans (Adams 1995a, Price and Fenwick 1985). The concentration of coumestrol and other phytoestrogens is heavily influenced by the growth stage and environmental factors. Up to this point, biotic stress is known as the major determinant of biosynthesis of estrogenic plant metabolites. While coumestrol is often not detected in healthy vegetative lucerne, it is much greater in old leaves and prickly-covered coiled pods (Reed 2016).

Table 2. Levels of phytoestrogens in various feed sources (µg/100 g dry weight). The number in brackets shows the number of analyzed species. (Mazur et al. 1998, Mazur and Adlercreutz 1998)

Plant	Formononetin	Biochanin A	Daidzein	Genistein	Coumestrol	SECO	MAT
Forage legumes							
Lupinus mutabilis Lupin	23.1	0	trace	2420	0	3.1	0
Medicago spp. Smile sprouting alfalfa	0	0	3.7	11,5	trace	19.4	0.7
Trifolium spp. Smile sprouting clover (seed)	1270	381	178	323	5,4	13.2	trace
Trifolium pratense Red clover	22,300	20,400	12,200	4,010	105	trace	trace
Legumes							
Glycine max Soybean (4)	18.1-121	0-15	10,500-56,000	26,800-84,100	0-185	13-273	trace
Phaseolus vulgaris Kidney bean (11)	0-10.9	0-11.7	7-40	18-518	0-9.1	56-153	trace
Phaseolus lunatus Lima bean (3)	9-12.4	0-2.7	12.2-89	10.6-19.2	0-10	158-185	trace
Apios americana American groundnut (5)	0-3.6	3.6-7.8	0-18	108-811	trace	20.8-58.1	2.2-4.6
Cajanus cajan Pigeon pea (3)	5.1-26,1	10.4-219	12-27	190-737	trace	18.7-50.3	0
Cicer arietinum	94.3-215	838-3,080	11.4-192	69.3-214	0-5	6.4-8.4	0

Chickpea (3)							
Pisum sativum Pea (4)	0-10	3-5.6	3.7-11.3	0-22.8	trace	2.8-12.8	trace
Trigonella foenumgraecum Fenugreek	trace	trace	10.2	9.8	0	8.6	trace
Vicia faba Broad bean (2)	6.3-39	trace	15.8-31.8	trace	0	26-31.8	0-131
Vigna mungo Black gram (3)	0-2	0-81.1	6.9-35.9	0-60.3	0-9.5	45.7-240	70.8-262
Vigna unguiculata Cowpea (2)	0-5.5	0-7.7	20.5-30.3	11.4-55.7	0-7.7	195-196	0
Vigna radiata Green gram	7.5	14.4	9.7	365	trace	172	0
Arachis hypogaea Groundnut	6.8	6.5	49.7	82.6	0	333	trace
Lens culinaris Lentil (2)	7.5-10.7	0-7,1	3.3-10.4	7.1-18.8	0-6.8	8.9-12.3	trace
Pueraria lobata Kudzu leaf	87	1,240	375	2,520	18.1	476	trace
Pueraria lobata Kudzu root	7,090	1,400	185,000	12,600	1,570	30.7	trace
Sophora japonica Japanese Pagoda Tree	322	830	319	265	9.9	1,590	38
Oilseeds and nuts							
Flaxseed			0	0		369,900	1,087
Flaxseed crushed			0	0		546,000	1,300
Sesame seed			140	14		90	608
Clover seed	1,270	381	178	323	5	13	4

Sunflower seed		8	14	610	0
Poppy seed		18	7	14	12
Peanut	31	58	64	298	trace
Cereal grains and their processed products					
Wheat (whole grain)		0	0	33	3
Wheat bran		4	7	110	0
Oat meal		0	0	13	0
Oat bran		0	0	24	155
Barley (whole grain)		14	8	58	0
Barley bran		6	16	63	0
Rye meal (whole grain)		0	0	47	65
Rye bran		0	0	132	167
Triticale (whole grain)		2	2	39	9
Triticale meal		2	1	21	11

SECO= Secoisolariciresinol, MAT = Metairesinol

2.4. Metabolism of phytoestrogens in ruminants

Like any drug or toxin, the intake of phytoestrogens is not equivalent to the biologically active amount. Dietary phytoestrogens undergo several metabolic processes such as transformation in the gastrointestinal tract, absorption, distribution, biotransformation and excretion in bile, faeces, urine and milk (Grgic et al. 2021). Effects of phytoestrogens depend on many factors. Each species and sex are variously susceptible to them. The duration of exposure, the amount of exposure and the timing during reproductive development and cycle are also important (Nielsen and Williamson 2007).

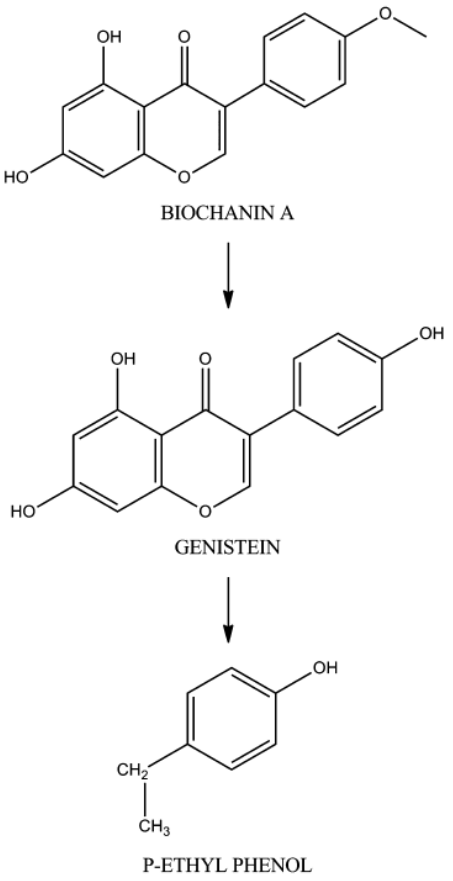


Figure 7. Ruminal metabolism of biochanin A to P-ethyl phenol (Mostrom and Evans 2018)

Most phytoestrogens appear in plants as biologically inactive glycoside conjugates with carbohydrates or glucose moieties. This means that after feeding phytoestrogen-containing fodder, further metabolization in the gastrointestinal tract for exerting E2 like activity is necessary (Nielsen and Williamson 2007). The most important chemical reaction is the hydrolysis of the conjugation between the phytoestrogen and its carbohydrate or glucose part, resulting in a biologically active aglycone. Accountable for this process, taking place in rumen and/or intestine, are β-glycosidase enzymes produced by gut microorganisms or intestinal epithelial glands (Kelly et al. 1993). Of metabolites tested for, this step is necessary for the isoflavonic glycosides daidzin, genistin, glycitin and ononin.

Ruminal microflora converts phytoestrogens, mainly isoflavones, to other metabolites. Biochanin A is demethylated to genistein and genistein by ring cleavage to the non-estrogenic compound para-ethyl phenol and organic acids (Figure) (Mostrom and Evans 2018). Most of biochanin A and genistein are metabolized in the reticulo-rumen and just a small fraction of these compounds are recovered in the omasum (Njåstad et al. 2014).

Formononetin is demethylated to daidzein and daidzein by hydrogenation and ring fission to the more potent estrogenic compound equol (Figure) (Benassayag et al. 2002, D'Alessandro

et al. 2005, Setchell and Clerici 2010a, 2010b). Formononetin can also be reduced to O-methyl equol or can be metabolized to O-DMA. After adaption to phytoestrogens a large population of rumen microbes are capable of metabolizing them (Mostrom and Evans 2018). This means that the estrogenic activity of biochanin A and genistein is limited to a few days of initial exposure when the rumen microbes need time to adapt to converting them into non-estrogenic metabolites P-ethyl phenol and phenolic acid. The ingestion of formononetin and daidzein may lead to the metabolization of compounds with higher (i.e. equol) or lower (i.e. O-DMA) estrogenic activity (Mostrom and Evans 2018).

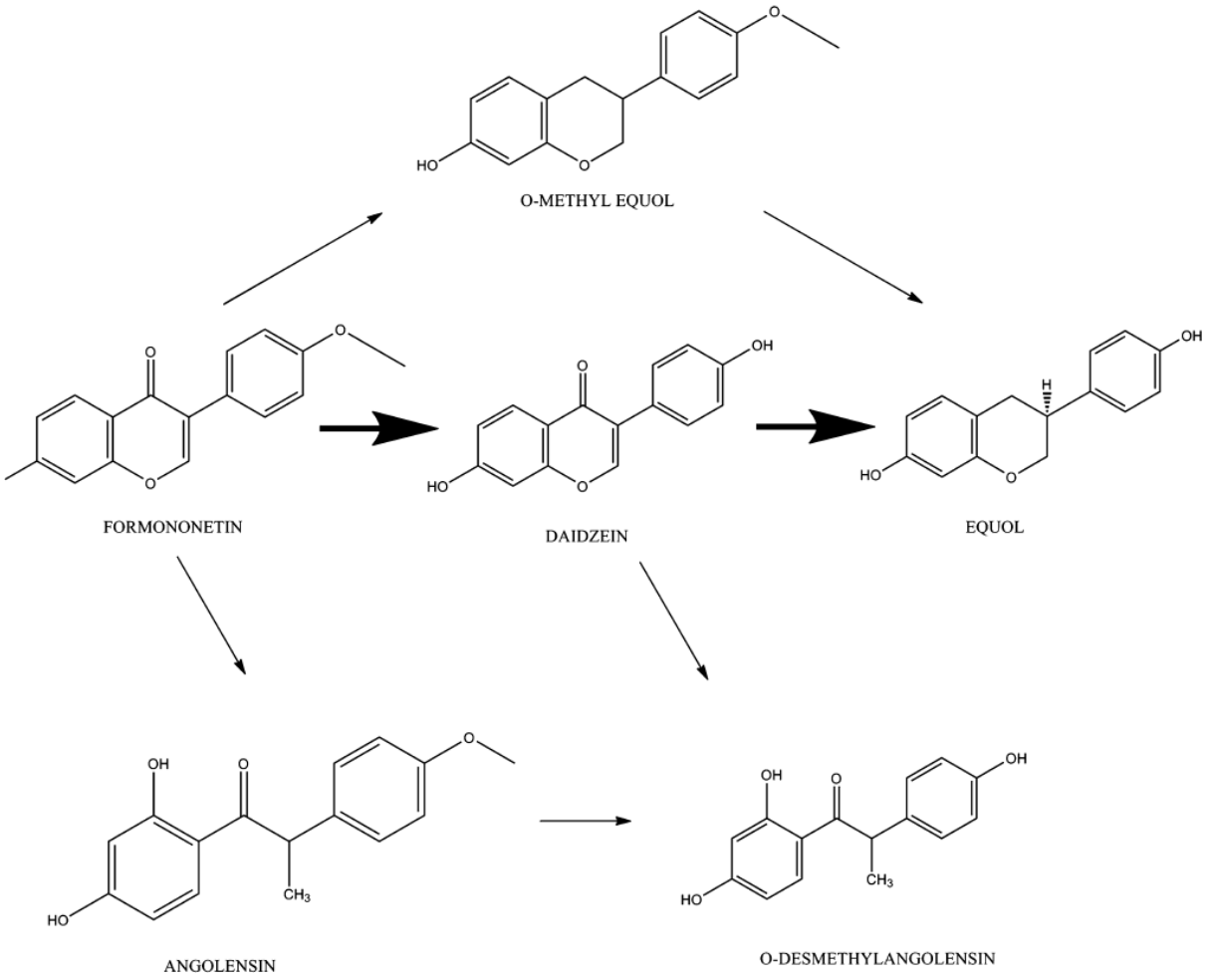


Figure 8. Metabolic pathway of formononetin via daidzein to equol in the rumen (Mostrom and Evans 2018)

The plant lignans MAT, SECO and their glycosides are coverted to enterodiol and enterolactone by microbes in the intestinal tract. Enterodiol and enterolactone are active, estrogenic mammalian lignans (Wang 2002). Only the active metabolites and unconjugated forms (aglycones) seem to exert estrogenic activity in animals. Re-conjugation of aglycones

can happen in the epithelial cells of the elementary tract during absorption (Lundh 1995). After absorption most of the free circulating plant-based estrogens are re-conjugated to glucuronic acid and a minor fraction to sulphuric acid, which is very important for the detoxification of phytoestrogens. A small amount of free, hydrolysed compounds are absorbed through the intestinal mucosa and reach the blood circulation unconjugated. They are then conjugated by liver, hepatic UDP-glucuronosyltransferases and sulfotransferases, and other tissues including kidney (Branca and Lorenzetti 2005, Lundh 1995). When entering the systemic circulation, additional chemical reactions including methylation, hydroxylation, demethylation, chlorination, iodination and nitration may happen (Benassayag et al. 2002, D'Alessandro et al. 2005). The absorption of phytoestrogens in cattle occurs very fast. Free and conjugated formononetin and daidzein reach their maximum plasma level within one hour after feeding (Lundh 1995). Also, the health status influences the concentration of active metabolites in the blood plasma of cows. Experimentally induced metritis and mastitis showed increased isoflavone absorption, biotransformation and metabolism (Kowalczyk-Zieba et al. 2011). Thus, cows with induced inflammation are more exposed to active isoflavone metabolites than healthy cows. Mammalian isoflavones and lignans can be found in bile, serum and urine following phytoestrogen intake. Like endogenous estrogens, phytoestrogens undergo enterohepatic circulation (Lundh 1995). The main route of excretion of lignans and isoflavonoids is through feces (Njåstad et al. 2014).

Phytoestrogens exert their effects primarily by binding to ER. Two types of ER are known in mammals, Erα and Erβ. Erα is predominantly expressed in endometrium, ovarian stroma cells, efferent duct epithelium and the hypothalamus. Erβ is featured in kidney, brain, heart, bone, lungs, prostate, intestinal mucosa and endothelial cells. Phytoestrogens have a lower affinity for Ers than E2 and most of them exhibit an approximately 30-fold higher affinity for Erβ than Erα (Pettersson and Gustafsson 2001, Turner et al. 2007, Whitten and Naftolin 1998). Chemical structures are key elements in binding to ER. A phenolic ring is essential for binding, low molecular weight is beneficial, and the distance between two hydroxyl-groups at the isoflavones nucleus shall be as similar to estradiol as possible (Yildiz 2006). With the aid of a receptor binding affinity test, the affinity of different phytoestrogens regarding the E2 sub-types was studied. The estrogenic potency of phytoestrogens differs between the two sub-types and are ranked as below mentioned: E2 > zearalenone = coumestrol > genistein > daidzein > apigenin = phloretin > biochanin A = kaempferol = naringenin > formononetin = ipriflavone = quercetin = chrysin for Erα. And E2 > genistein = coumestrol > zearalenone > daidzein >

biochanin A = apigenin = kaempferol = naringenin > phloretin = quercetin = ipriflavone = formononetin = chrysin for Erβ (Kuiper, George G. J. M. et al. 1998).

2.5. Negative impacts of Phytoestrogens on ruminants

In 1995 Norman R. Adams discussed the effects of phytoestrogens on fertility in ewes. On display were two forms of infertility, temporary and permanent. Both often occur without apparent clinical signs and can only be detected by measurement of phytoestrogens in feed or their effects on animals. Temporary infertility results from actions of estrogens resembling the activating effects of estrogen in most mammal species. Permanent infertility originates from cervical changes, which are similar to the effects of estrogen described in other species treated during organogenesis. It is believed that in ewes, these effects can be triggered after organogenesis by prolonged exposure during adult life (Adams 1995b). The levels of metabolic and nutritional hormones can generate histological changes in the cervix produced by lengthy treatment with estrogen. The ongoing influence of estrogen results in the cervix showing uterus-like changes under the microscope. The hypothesis is that, owing to nutritional circumstances, the hormonal milieu in ewes simulates hormonal patterns, which are usually experienced by foetal lambs intrauterine, thereby allowing the adult cervix to give an organizational retort to estrogen. Although optimal ovarian function, the defeminized cervix leads to a worsening ability to store spermatozoa and a reduced conception rate (Adams 1995a, 1995b). Since then, no publication on the same hypothesis was found.

Adams (1995a, 1995b) also discussed the effect of phytoestrogens on cows. Diets rich in alfalfa and red clover may lead to impaired ovarian function, often accompanied by increased embryonic loss and reduced conception rates. Cows and castrated bulls may experience epithelium hypertrophy of the mammary glands and secret a clear or milky fluid. The clinical signs of phytoestrogen exposure in cows are similar to those in cows with cystic ovaries. The infertility is temporary and is expected to resolve within weeks or months after the removal of the estrogenic feed (Kallela et al. 1984). While subclinical concentrations are suggested to play an important role in humans, effects on cattle have not been described yet (Adams 1995a).

2.6. Influence of farming systems on phytoestrogen concentrations

A group of Finnish scientists compared the concentration of equol in milk and plasma of twelve Finnish Ayrshire cows fed with six different types of fodder (Mustonen et al. 2009). They also analysed the concentration of various isoflavones in silage. The different silages were offered *ad libitum* and supplemented with an equal amount of concentrate. The two grass silages were primary growth of timothy (*Phleum pratense*), one cut early on June 15th and the other late on June 30th. The four red clover (*Trifolium pratense*) silages were primary growth cut early on July 1st and the other late on July 14th, with their regrowth being harvested on August 24th. While grass silages did not contain any investigated isoflavones, the red clover, especially the ones with shorter vegetation period, contained high isoflavones (red clover early: 10.41 g/kg DM; regrowth of red clover late: 11.78. g/kg DM). Cows fed with silages rich in isoflavones showed significantly higher contents of equol in milk and plasma. The study showed that the growing period of red clover has a substantial influence on its own isoflavone amount and, as a result, on the cows' equol concentrations in plasma and milk.

Scandinavian scientists analysed the milk content of phytoestrogens in 28 Norwegian Red dairy cows fed with red or white clover grass silages with or without 10 kg of concentrate (Steinshamn et al. 2008). Cows fed with red clover silage showed higher content of isoflavones in milk, while cows fed with white clover silage showed a greater intake of lignans. However, when supplemented with concentrate, both groups showed reduced isoflavone concentrations in milk, while the mammalian lignans enterodiol and enterolactone gained ground.

Another group of Scandinavian scientists investigated the metabolism of phytoestrogens in four Norwegian Red dairy cows fed with four different kinds of silage (Njåstad et al. 2014). The first silage was organically managed second-year timothy and red clover ley. The second one was organically managed long-term grassland at six years of age. The other two were conventionally managed perennial ryegrass (*Lolium perenne*) and timothy ley. The concentration of isoflavones in organically managed short-term timothy and red clover was 35 to 98 times higher than in the other three silages, while conventionally managed timothy contained largely lignans. Outcome of the study was that phytoestrogens in milk can be manipulated through fed fodder (Njåstad et al. 2014).

Norwegian and Danish scientists compared the impact of organically and conventionally managed short-term and long-term grassland on phytoestrogens in bulk-tank milk (Adler et al. 2015). Fourteen organic dairy farms were paired with 14 conventional dairy farms, all of them

varying in grassland management. The analysis of botanical composition showed that organically managed grassland contained the most considerable proportion of red clover resulting in more equol in bulk-tank milk than compared conventional farms. They further showed that feeding short-term grassland resulted in a greater amount of equol in milk than long-term grassland. Although they had the biggest amount of red clover in their herbage, organically managed dairy farms with short-term grassland management didn't show signs of fertility problems.

2.7. Aims and Hypothesis

This diploma thesis aims to determinate the occurrence of phytoestrogens in rations of Austrian dairy farms that operate under different conditions with a focus on feeding systems. In addition, conventional and organic dairy farms working under similar conditions are compared regarding their phytoestrogen concentration in rations.

The hypothesis of this work is, that the TMR of organic farms contains a significant higher amount of phytoestrogens than the rations of conventional farms.

3. Materials and Methods

This work is part of the project D4Dairy (Digitalisation, Data integration, Detection and Decision support in Dairying), which evaluates relevant information on feed quality in 98 Austrian dairy farms located in 3 different states: Lower Austria (n=31), Upper Austria (n=51), and Styria (n=16) (Penagos-Tabares et al. 2021, Penagos-Tabares et al. 2023, Penagos-Tabares, Khiaosa-ard et al. 2022, Penagos-Tabares, Sulyok et al. 2022). These farms were part of the target farms that met the selection criteria in terms of location (Styria, Lower and Upper Austria) and farm size (number of lactating cows >50). They also had to actively record their data on animal health, production and reproduction. With the farmers' consents, the farms were included in this research project, performing feed sampling and data collection in each farm. The farms included in the study presented an average herd size of 59 ± 15 standard deviation (SD) lactating cows per farm, fluctuating from 32 to 140 and used automatic milking systems (AMS). Of these 98 farms, 89 (90.8%) farms were managed conventionally and the remaining nine (9.1%) organically. The feed sampling took place from June to September 2020, the preparation for further analysis was performed from October to November 2020 and the liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis happened during the first two months of 2021.

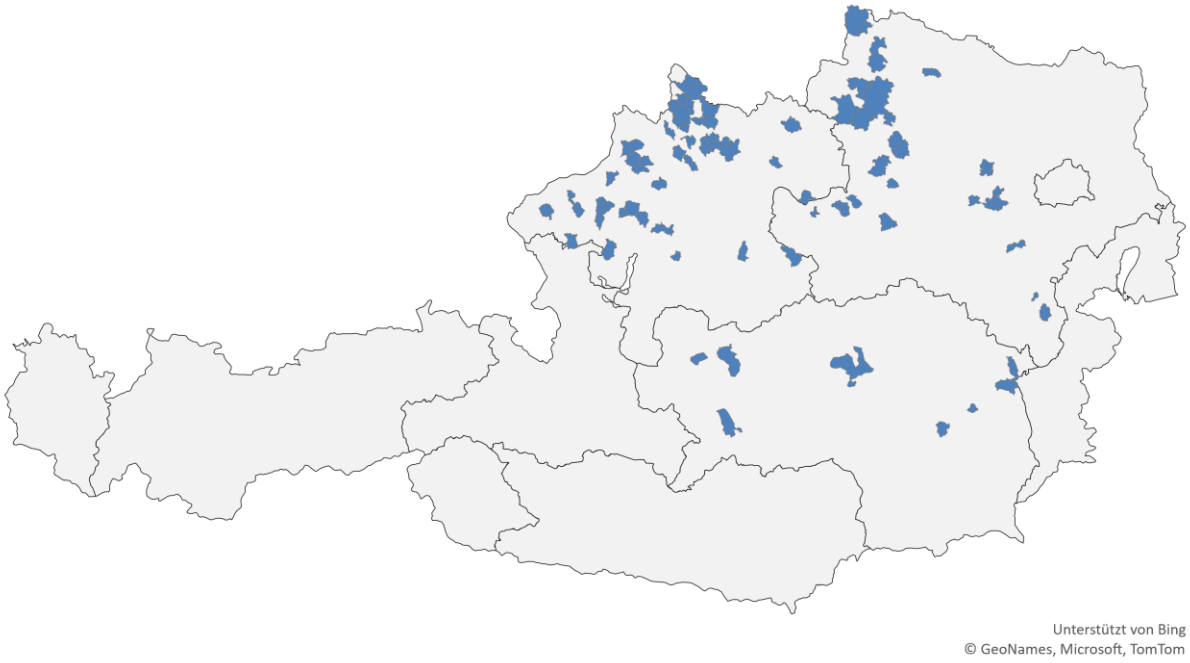


Figure 9. Locations of surveyed dairy farms (n=98)

3.1. Feed sampling

Partial mixed ration (PMR) and total mixed ration (TMR) samples were collected manually from 20-30 different spots of the feeding table immediately after serving. Collection of samples after the cows fed from it would have falsified the data because of cows' feed sorting behaviour. It was necessary to collect the samples carefully and homogenously, avoiding selection of big particles, to obtain representative samples. The collected sample was placed on a 1x1 m plastic film and gently mixed by hand. Subsequently, the samples were quartered and one part with a total weight of about 1 kg were vacuum packed in a plastic bag. Until further processing, the samples were stored at -20°C to avoid microbial growth and activity during storage, which could modify the original dietary concentrations of phytoestrogens and mycotoxins of the samples.

In the case of PMR, samples of additional fed concentrate were collected from AMS, automatic feeders or silos. In case the farm used more than one kind of concentrate, the sample was mixed according to the fed proportions. Following this, about 1 kg of representative sample of additional fed concentrate was vacuum sealed in a plastic bag.

3.2. Sample preparation

Before multi-phytoestrogen analysis, the samples were prepared by using an previously published method (Penagos-Tabares, Khiaosa-ard et al. 2022). PMR and TMR contained considerable levels of moisture and needed to be dried. Accordingly, after thawing for 24 h at room temperature, each sample was divided into two aluminium trays, weighed, and subsequently dried in an air oven at 65 °C for 48 h. The air-dry weight was used for later calculation. Subsequently, the dried PMR, TMR and concentrate were ground to a final particle size of 0.5 mm with the cutting mill (SM 300, Retsch GmbH, Germany) at 1,500 rpm. First, the sample was ground with a 2-mm sieve followed by grinding using a 0.5-mm sieve. Part of the sample that remained coarse was further processed in the ultra-centrifugal mill (ZM 200, Retsch GmbH, Germany) at 10,000 rpm using a 0.5-mm sieve. The ground samples from both mills were collected and pooled in a plastic bag and properly mixed. After grinding PMR and concentrate samples of each farm were pooled accordingly to their estimated dry matter intake, to obtain a representative sample of the complete ration of each farm. Pooling was not necessary for the TMR samples. To prevent cross contamination both mills and all the worktops were thoroughly cleaned with a vacuum cleaner between the processing of each sample.

3.3. Liquid Chromatography with tandem mass spectrometry

For further analysis the same source as for sample preparation was used (Penagos-Tabares, Khiaosa-ard et al. 2022). Five g (±0.01g) of each farm sample (n=98) were put into 50 ml polypropylene conical tubes (Sarstedt, Germany) and 20 ml of the extraction solvent (acetonitrile/water/acetic acid 79:20:1, *v/v/v*) was added. Extraction was performed using a rotary shaker (GFL 3017, GFL, Germany) in a horizontal position at 180 rpm for 90 min. Subsequently the tubes were sedimented in a vertical position for 10-15 min. Posteriorly, 500 µl of supernatant was diluted with an equal amount of dilution solvent (acetonitrile/water/acetic acid 20:79:1, *v/v/v*) into autosampler vials. Five µl of diluted raw extracts were then injected into the Qtrap 5500 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source, which was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany) as stated in (Sulyok et al. 2020). Quantification was accomplished from external calibration by serial dilutions from multiple analytes. Through spiking experiments determined apparent recoveries were corrected (Steiner et al. 2020).

Measurements were performed at the Department of Agrobiotechnology (IFA-Tulln) at the University of Natural Resources and Life Sciences Vienna (BOKU) in Tulln, Austria. The analytic method has been described in detail previously (Steiner et al. 2020, Sulyok et al. 2020). 800 metabolites were tested for with LC-MS/MS and 155 substances were detected. Ten from the 155 substances were phytoestrogens and used for this thesis work. These were biochanin A, coumestrol, daidzein, daidzin, formononetin, genistein, genistin, glycitein, glycitin and ononin. Nine of them belong in the family of isoflavones, part of them being glycosides and the others being aglycones. Coumestrol represents the coumestans. Table 5 in the Annex shows the apparent recovery, the limit of detection (LOD) and the limit of quantification (LOQ) of each target metabolite from the LC-MS/MS analysis. LOD describes the minimal number of phytoestrogens needed, to reliably differ whether they occur or occur not in the sample. LOQ represents the minimal amount of phytoestrogen that can be quantified with stated accuracy and precision. All data above LOQ is considered quantified.

3.4. Statistical Analysis

There were two separate evaluations performed in this thesis. The first part dealt with the surveyed data from all 98 farms on the occurrence and concentration of phytoestrogens in dairy rations. The second part compared the effect of farm management (conventional vs.

organic) and the nine organic farms were opposed to nine comparable conventional farms. The deciding factors for the selection of conventional farms were geographic proximity and a similar share and choice of forages in the diet. This means a relatively low use of maize silage in conventional farms compared to other conventional farms. Both groups were balanced by the number of farms per province: per group there were four farms from Lower Austria, two farms from Styria and two farms from Upper Austria. The Mann-Whitney Test was used to determine whether the two groups showed a significant difference in their phytoestrogen content.

Descriptive statistics of occurrence and concentration values were executed using data above LOD. Values of phytoestrogens below LOD were considered not detectable. Concentrations > LOD and < LOQ were calculated as LOQ/2. The results are based on dry mater (DM) basis and presented in µg/kg on a logarithmic scale whenever applicable. Figures were produced using Microsoft Excel (Microsoft Corporation, Microsoft Excel, 2018) and the heat map of the co-occurrence matrix of phytoestrogens was created with GraphPad Prism (Prism version 9.1, GraphPad Software, USA). The performance values of the analytic method (including LODs, LOQs and apparent recoveries) are presented in Table 5 in the annex. Information on diet composition (main dietary ingredients) and milk yield were obtained by questionnaire-guided personal interview.

Comparison between conventional and organic farm groups was performed according to the Mann-Whitney test using PROC NPAR1WAY of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). Simple regression of forage inclusion level and dietary phytoestrogen concentration was done using PROC REG of SAS.

4. Results

4.1. Occurrence and levels of phytoestrogens in diets of Austrian dairy cows

Ten phytoestrogens were detected (biochanin, coumestrol, daidzein, daidzin, formononetin, genistein, genistin, glycitein, glycitin and ononin). Their respective occurrence, average ± standard deviation, median, minimum and maximum concentration are presented in the Annex (Table 6). Regarding the occurrences, all samples contained at least four phytoestrogens (Figure 10). Biochanin, daidzein and genistein were ubiquitous among the samples. Coumestrol, daidzin, genistin and glycitin showed occurrences between 70% and 90% of the samples. Onionin, formononetin and glycitein were detected in 52%, 21% and 10% respectively.

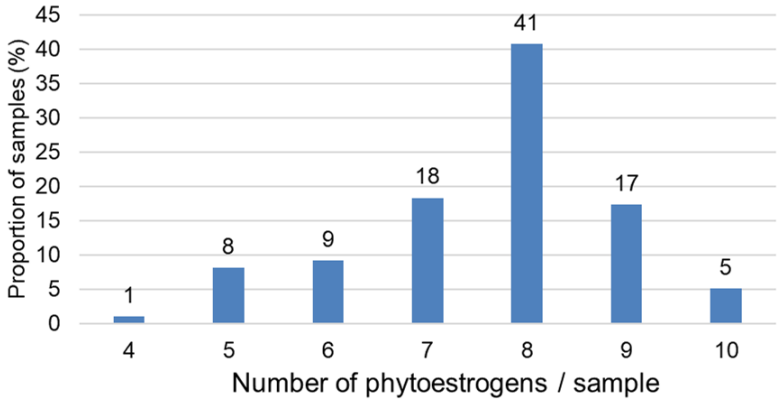


Figure 10. Number of frequency of phytoestrogens in Austrian dairy cows in percent

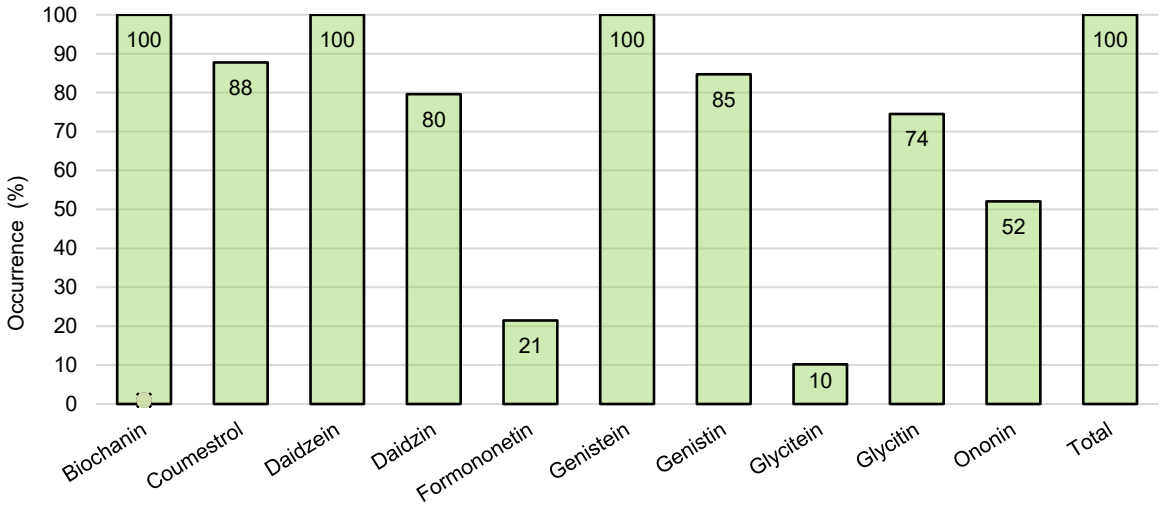


Figure 11. Occurrence of phytoestrogens in diets of Austrian dairy cows

Concentration of detected phytoestrogens of positive samples are illustrated in Figure 12. The bottom light blue and top black dots indicate the minimum and maximum concentrations respectively and the black cross shows the average concentration (bold numbers, right side). The line on the bottom of the boxplot marks the 25th percentile and on top the 75th percentile, the line in between illustrates the median concentration. The number positioned on the left side next to the grey dot on the median line is the median value. On average, the samples contained 83,224 µg/kg DM of total phytoestrogens, ranging from 4,260 µg/kg DM to 411,420 µg/kg DM. Formononetin showed the highest concentration among all detected phytoestrogens with a mean concentration of 114,017 µg/kg. The next highest concentration was biochanin with an average of 24,706 µg/kg (mean: 1,101 µg/kg; max: 52,051 µg/kg). Daidzein, daidzin, genistein and genistin showed similar ranges with average concentrations from 4,659 µg/kg to 13,042 µg/kg DM. Coumestrol showed the least concentration with an average of 645 µg/kg and a range from 19 µg/kg DM to 8,286 µg/kg per sample. This phytoestrogen also had the widest range in concentration, with the maximum value being 436 times the minimum concentration.

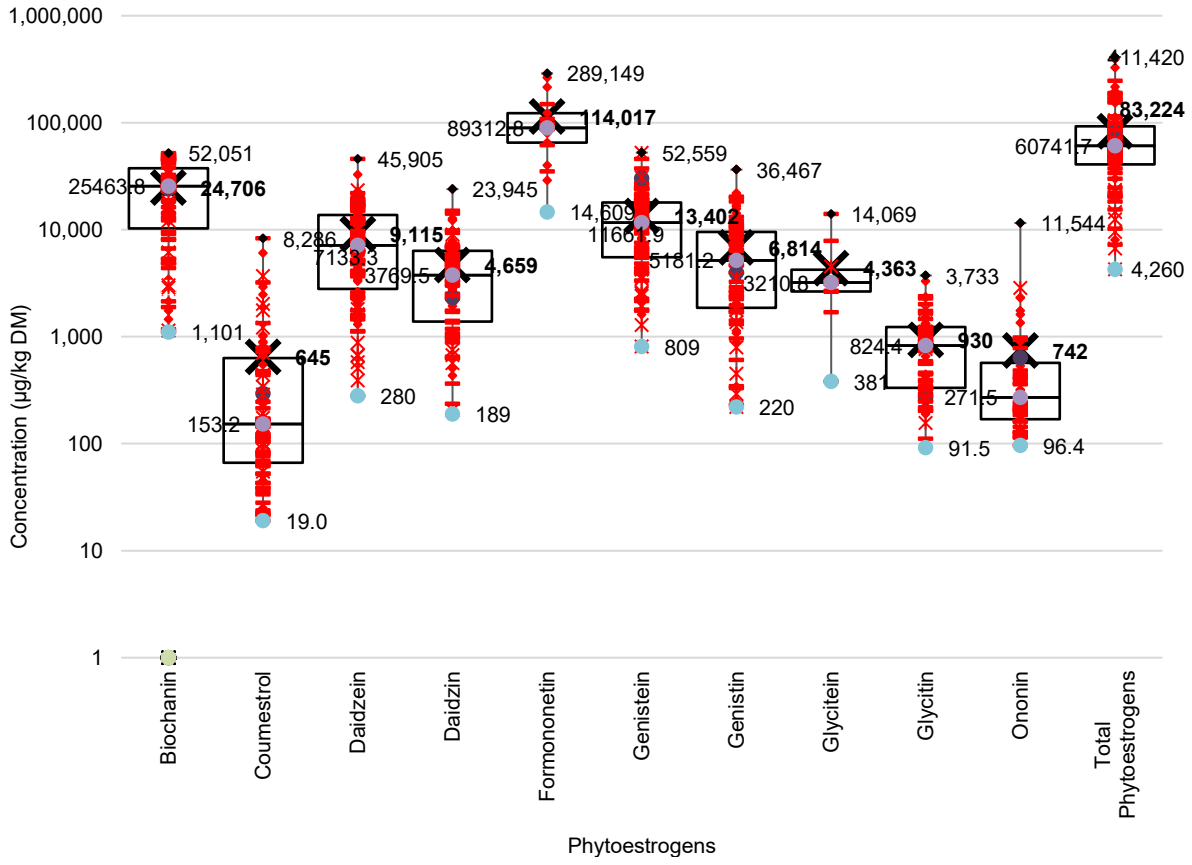


Figure 12. Levels of phytoestrogens in diets of Austrian dairy cows

The samples contained between four to ten phytoestrogens per sample with an average of 7.6 per sample. Figure 10 illustrates the number of phytoestrogens per sample. Around 81% of the samples contained ≥ seven phytoestrogens per sample. The heatmap in Figure 13 shows the co-occurrence of phytoestrogens in the tested samples. Biochanin, daidzein and genistein were detected in all samples and therefore their co-occurrence with all other phytoestrogens was 100%. The majority of samples positive for glycitin also contained coumestrol (co-occurrence of 67%). All the samples containing daidzin (80%) also tested positive for genistin. Formononetin (21%) and glycitein (10%) were not only the least occurring phytoestrogens but also never occurred together in the same sample. While ononin was detected in 52% of the samples it frequently co-occurred with formononetin but rarely with glycitein. Accordingly, 19 from 21 samples testing positive for formononetin contained ononin as well. At the same time just two from ten samples testing positive for glycitein contained ononin.

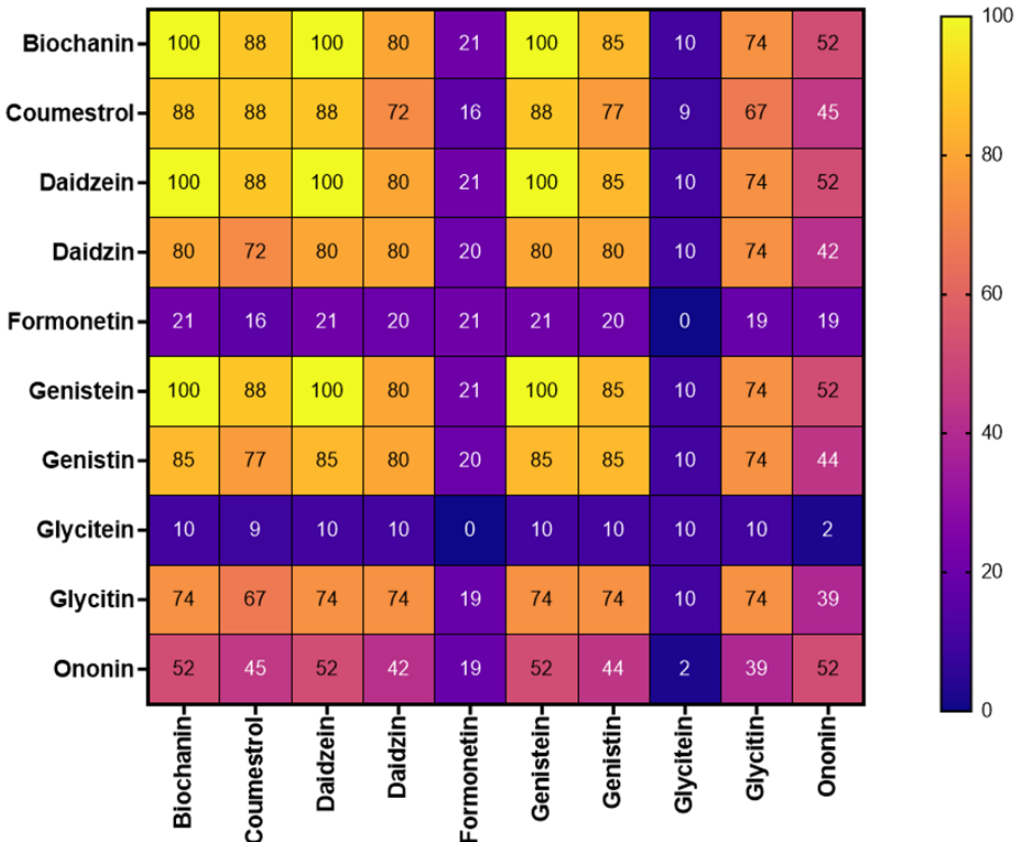


Figure 13. Co-occurrence analysis of phytoestrogens in Austrian dairy cows in percent (n=98)

4.2. Comparison of dietary levels of phytoestrogen in organic and conventional dairy farms

As shown in Table 3, the nine organic farms was twice the concentration of total phytoestrogens in µg/kg DM compared to conventional farms but were not deemed statistically significant (P = 0.190). Taking a closer look at the samples, this is due to high variations in concentration in organic samples.

Formononetin was the most abundant phytoestrogen in the organic samples followed by biochanin, while it was the other way around for conventional samples. According to the Mann-Whitney Test Formononetin was not up to interpretation because only three organic and two conventional samples tested positive for it and therefore deemed invalid (P = invalid). Daidzin (P = 0.280), genistin (P = 0.334) and glycitin (P = 0.242) were found in higher concentrations in conventional farms but differences turned out to be not significant. None of the investigated farms tested positive for glycitein.

Nevertheless, there were distinct differences in four out of the remaining nine phytoestrogens. All chosen farms tested positive for coumestrol (P = 0.008) and all organic farms tested positive for ononin (P = 0.006). Both metabolites were significantly more often found in organic samples. Daidzein (P = 0.002) and Genistein (P < 0.001) were found in all samples and also significantly higher in organic farms.

The numbers in Table 4 show that all farms feed a similar amount of DM via concentrate (organic: 32.59%; conventional 31.73%) and forage, respectively. However, the use and inclusion levels of grass silage and maize silage differed between organic and conventional farms. All 18 farms incorporated grass silage in their ration, while eight out of nine conventional and just one out of nine organic farms used maize silage. As a result, the average dietary ration of the organic farms consists of 62.32% grass silage in DM and in conventional farm 47.28% in DM. Regarding dietary levels of maize silages, conventional farms averaged 18.26%, whereas in diets of organic farms it was only 2.06%. The Mann-Whitney Test confirmed the significantly higher use of grass silage in organic farms (P = 0.032) and higher use of maize silage in conventional farms (P = 0.002).

Table 3. Comparison of dietary levels of phytoestrogens in samples from organic and conventional dairy farms

Metabolite	Organic farming (n=9)					Conventional farming (n=9)					Mann Whitney Test		
	% positive	Concentrations (µg/kg)				% positive	Concentrations (µg/kg)						
		Average ± SD	Median	Range			Average ± SD	Median	Range		Pr ≥|S - Mean|	Pr > |Z|	Higher in
Biochanin	100	39,617 ± 12,030	47,387	23,877 - 51,098		100	33,865 ± 13,115	33,154	12,102 - 49,498		0.374	0.389	No difference
Coumestrol	100	1,284 ± 1,100	895	148 - 3,685		100	426 ± 791	118	25 - 2,472		0.008	0.020	Organic
Daidzein	100	14,441 ± 8,188	13,762	5,768 - 32,722		100	4,258 ± 3,680	3,400	507 - 12,069		0.002	0.010	Organic
Daidzin	56	2,543 ± 3,148	1,111	0 - 7,814		78	4,861 ± 4,823	4,915	0 - 15,033		0.280	0.296	No difference
Formononetin	33	66,817 ± 106,445	0	0 - 263,672		22	19,501 ± 38,701	0	0 - 89,067		*Invalid*	*Invalid*	*Invalid*
Genistein	100	25,913 ± 15,705	20,599	11,662 - 52,559		100	6,931 ± 3,365	7,383	1,943 - 12,183		<0.001	0.003	Organic
Genistin	67	4,319 ± 5,498	1,136	0 - 14,235		78	7,206 ± 6,571	9,167	0 - 20,356		0.334	0.340	No difference
Glycitin	56	402 ± 489	224	0 - 1,229		78	794 ± 770	826	0 - 2,389		0.242	0.258	No difference
Ononin	100	1,119 ± 977	604	162 - 2,858		78	182 ± 136	186	0 - 379		0.006	0.017	Organic
Phytoestrogens	100	156,456 ± 129,322	93,999	48,971 - 403,213		100	78,023 ± 49,810	64,486	14,639 - 154,660		0.190	0.203	No difference

Pr > |Z| = The P-value corresponding to the two-sided test based on Student's t distribution

Pr ≥ |S - Mean| is the exact two-sided P-value

Comparison of Formononetin is deemed invalid due to very small number of positive samples in both famring systems

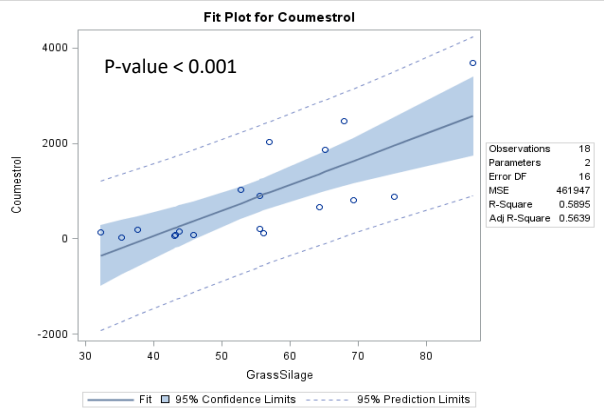
Table 4. Feed composition in organic vs conventional samples

Diet parameter	Organic farming (n=9)					Conventional farming (n=9)					Mann Whitney Test		
	% positive	% diet DM				% positive	% diet DM						
		Average ± SD	Median	Range			Average ± SD	Median	Range		Pr ≥|S - Mean|	Pr > |Z|	Higher in
Maize silage	11	2.06 ± 6.17	0	0 - 18.52		89	18.26 ± 11.03	20.36	0 - 35.78		0.002	0.008	Conventional
Grass silage	100	62.32 ± 13.12	56.96	43.74 - 86.80		100	47.28 ± 12.71	43.24	32.20 - 67.89		0.032	0.049	Organic
Straw	56	1.94 ± 2.14	1.62	0 - 4.99		67	2.23 ± 2.69	1.24	1.24 - 7.37		0.292	0.929	No difference
Hay	33	1.09 ± 2.48	0	0 - 7.52		22	0.51 ± 1.03	0	0 - 2.73		*Invalid*	*Invalid*	Invalid
Concentrate	100	32.59 ± 12.15	28.44	13.20 - 48.74		100	31.73 ± 8.89	29.20	20.95 - 46.00		0.730	0.728	No difference

Pr > |Z| = The P-value corresponding to the two-sided test based on Student's t distribution

Pr ≥ |S - Mean| is the exact two-sided P-value

Regression analysis confirmed a positive correlation between the inclusion level of grass silage and coumestrol in these 18 farms (P < 0.001) (Figure 14-17). A positive correlation of grass silage level with other phytoestrogens differing between organic and conventional farms including genistein (P = 0.256), ononin (P = 0.211) and daidzein (P = 0.180), according to in the Mann-Whitney Test, was found but did not reach significance. Despite being a phytoestrogen with high concentrations, the low occurrence formononetin was deemed invalid for Mann-Whitney test and regression analysis of the investigated conventional and organic farms.



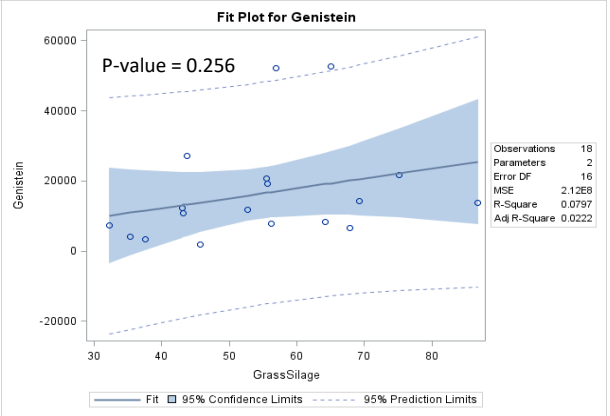
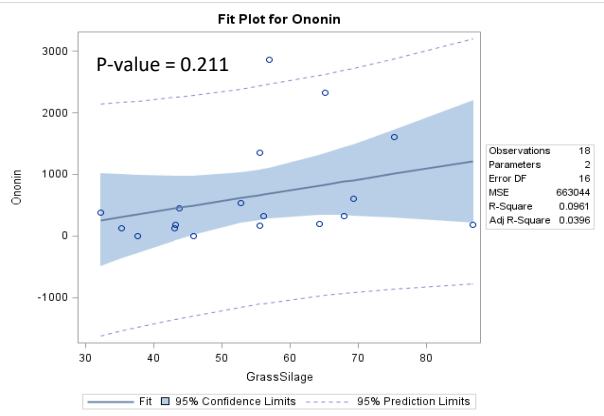


Figure 14. Correlation of % grass silage and content of coumestrol
Figure 15. Correlation of % grass silage and content of genistein



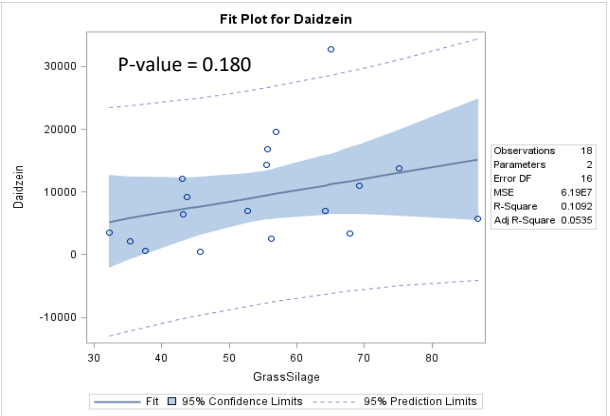


Figure 16. Correlation of % grass silage and content of ononin
Figure 17. Correlation of % grass silage and content of daidzein

5. Discussion

Looking at the results, the ubiquitous presence of phytoestrogens in dairy cows' diets is evident. The occurrence of the aglycones biochanin, daidzein and genistein was also ubiquitous. Interestingly, formononetin showed the highest concentration, when it was present, but it only occurred in 21% of the samples. This fact combined with the high estrogenic potency of its metabolite equol (Mostrom and Evans 2018) is a good reason to conduct further research. Another noticeable finding is, that glycitin (74%) was always accompanied by daidzin and genistin, daidzin (80%) always occurred together with genistin but genistin (85%) could occur without the other two glycosides. What agonistic and antagonistic effects individual phytoestrogens have on each other cannot be determined from this study. It could be worthwhile to investigate this in more detail, since there were no publications found.

On average the samples assessed in this work contained 83.2 mg/kg of total phytoestrogens. The organic farms averaged 156.5 mg/kg. When compared to previous studies, a heterogenous picture was painted. In 2008 (Steinshamn et al. 2008) reported about 227.6 mg/kg of phytoestrogens in white clover silage and 5,616.2 mg/kg in red clover silage respectively. More than 90% of phytoestrogen in the red clover silage was biochanin A und formononetin. Höjer et al. 2012 compared in another study five kinds of silages and the total phytoestrogen ranged from 215.5 mg/kg DM in long-term grassland silage to 5,245.2 mg/kg DM in 3rd-cut red clover grass silage. Mustonen et al. (2009) reported concentrations of 6,400 -11,780 mg/kg DM. All previous numbers derived from analysis of pure silage samples and not from dietary rations (i.e., TMR and PMR + additional fed concentrate) as assessed in this study. This makes comparison between the previous studies and this study difficult and is probably one of the reasons why all 98 samples were below previously reported concentrations.

In relation to the influence of phytoestrogens on fertility, it is stated that concentrations from 18 to >180 mg/kg of coumestrol and concentrations from 500 to 750 mg/kg of formononetin and biochanin A are associated with infertility in cattle (Mostrom and Evans 2018). In 1980 Lookhart reported that haylage causing estrogenic stimulation in cows when coumestrol was >37 mg/kg, resulted in bulling of steers, udder development, prolapsed vagina, cervix and rectum. Looking at the data of all 98 farms, the highest concentration of coumestrol was at 8.3 mg/kg DM, formononetin was most elevated at 289.1 mg/kg DM and the maximum concentration of biochanin A was at 52 mg/kg DM. All three farms were conventionally managed and not selected for comparison with organic farms. In fact, genistein was the only phytoestrogen out

of all ten that was found most frequently in an organic farm. According to the here presented results, the detected levels of single phytoestrogens do not implicate a serious risk for the reproductive performance of the respective herds. These values originate from representative samples of diets, meaning that some specific individual components can have higher concentrations of phytoestrogens. The fact that the highest concentrations were found on conventional farms may indicate the importance of ingredients other than grass silage, such as legumes and their by-products, and should be investigated further.

When comparing organic and selected conventional samples it becomes evident that while the average organic sample contains roughly double the concentration of phytoestrogens, this difference was not statistically significant. Regarding feed composition, the Mann-Whitney Test shows that organic farms feed a significantly higher amount of grass silage to their cows. It is reported that organic grass silage contains a higher number of phytoestrogens than conventional ones (Adler et al. 2015, Njåstad et al. 2014). Botanical composition (Adler et al. 2015) and age at harvest (Mustonen et al. 2009) play a key role in there as well. A Norwegian study compared organic and conventional silages with different botanical compostition (Njåstad et al. 2014): Conventionally managed perennial ryegrass (0.37 mg/kg DM) and timothy ley (0.49 mg/kg DM) contained significantly ($P < 0.001$) less coumestrol than organically managed short-term timothy and red clover in its second year (1.03 mg/kg DM) and long-term grassland with a high proportion of unsown species in its sixth year (1.48 mg/kg DM). Since further information concerning the sampled grass silage like age at harvest and grass population in this study is missing, it is difficult to identify the cause in this study. The graphs (Figure 14-17) showing the correlation between grass silage and the four phytoestrogens significantly higher in organic samples show a significant correlation between the amount of grass silage in TMR and coumestrol only. While daidzein, genistein and ononin are significantly higher in organic samples as well, there is no significant correlation of concentration with the amount of grass silage fed. This may indicate that coumestrol is present in all types of grass silage and is not strongly influenced by vegetation condition or other parameters not studied. There is a possibility that other phytoestrogens are also significantly higher in grass silage, but the type of grass silage needs to be more specified. Due to lack of data, correlation with other feed components was not possible. Soybeans are an important source of daidzein and genistein (Mazur and Adlercreutz 1998), a closer look at different concentrates could provide more information on phytoestrogens in concentrates.

Compared to isoflavones coumestans have been less researched (Gierus et al. 2012). While their metabolism in ruminants remains to be explored, it is already known that this does not seem to occur in the rumen (Njåstad et al. 2014). While present in all 18 samples, coumestrol has a small contribution on total phytoestrogen (Figure 14), but still has the most potent estrogenic activity of all known phytoestrogens to date (Pelissero et al. 1991). Understanding how this very potent phytoestrogen is metabolized in ruminants could contribute significantly to further understanding.

The interaction of isoflavones, flavones, stilbenoids, coumestans and lignans with other estrogenic substances, such as mycoestrogens, is a focal point of current research (Grgic et al. 2021). A recent publication suggests that co-contamination with mycotoxins, phytoestrogens, cyanogenic glucosides and other metabolites present in the diets of dairy cows leads to unexplored and unpredictable both antagonistic and synergistic toxic effects. Most of the compounds come with a wide range of biological and toxic activity, suggesting that the characterization of regulated contaminants in dairy cattle feed is only the tip of the iceberg of fungal and other environmental toxins (Penagos-Tabares, Khiaosa-ard et al. 2022).

5.1. Conclusion

Although four phytoestrogens (coumestrol, ononin, daidzein, genistein) were found significantly more frequently in samples from organic farming, the hypothesis of a higher total amount of phytoestrogens in samples from organic farming compared to conventional farms cannot be confirmed. However, a significantly higher proportion of grass silage was used in the organic samples, and when the four phytoestrogens found significantly more frequently in the organic samples were correlated with them, coumestrol showed significancy ($P < 0.001$). Compared to the literature, the levels found do not pose a serious risk to the reproductive performance of the respective herds.

6. Summary

This work is part of the project D4Dairy (Digitalisation, Data integration, Detection and Decision support in Dairying) [sub-project 2.5: Mycotoxin Detection and Implications for Dairy Performance - Screening mycotoxin contamination in feeds as causal agent of infertility and poor health in dairy cattle], which evaluates relevant information on feed quality in 98 Austrian dairy farms located in 3 different states: Lower Austria (n=31), Upper Austria (n=51), and Styria (n=16). Samples of basal feed (mostly partial mixed ration, but also forage and total mixed ration), as well as concentrate feeds, were representatively sampled and prepared for analysis by drying, grinding and pooling. Feeds of each farm were pooled according to the ration concentrate/basal feed and the levels of 10 phytoestrogens (biochanin, coumestrol, daidzein, daidzin, formononetin, genistein, genistin, glycitein, glycitin and ononin) of the ration were analysed by a liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis. A statistical analysis to characterize the occurrence and concentration of the identified phytoestrogens overall and conventional versus organic farms was performed.

Each sample contained at least four different phytoestrogens, five samples tested positive for all ten. Biochanin, daidzein and genistein were ubiquitous among the samples. The total phytoestrogen in samples ranged from 4,260 µg/kg DM to 411,420 µg/kg DM and averaged 83,224 µg/kg DM. Additionally nine organic farms were opposed to nine comparable conventional farms. The inclusion criteria for selection of farms were geographic proximity and a similar share and choice of main forages in the diet. Four phytoestrogens (coumestrol, ononin, daidzein, genistein) were found significantly more often in organic samples. When correlated with the significantly bigger amount of grass silage used in organic farms, only coumestrol showed significance ($P < 0.001$). In comparison to the literature, the detected levels of single phytoestrogens do not implicate a serious risk for the reproductive performance of the respective herds. The present values originate from representative samples of diets, therefore some specific individual components could have contained higher concentrations of phytoestrogens. The interaction of phytoestrogens and other estrogenic substances (like mycoestrogens) is a focal point of current research and still is at the very beginning.

7. Zusammenfassung

Diese Arbeit ist Teil des Projekts D4Dairy (Digitalisierung, Datenintegration, Detektion und Entscheidungsunterstützung in der Milchwirtschaft) [Teilprojekt 2.5: Mykotoxinnachweis und Auswirkungen auf die Milchleistung - Überprüfung der Mykotoxinkontamination in Futtermitteln als Kausalfaktor für Unfruchtbarkeit und schlechte Gesundheit bei Milchvieh], das relevante Informationen zur Futterqualität in 98 österreichischen Milchviehbetrieben in 3 verschiedenen Bundesländern auswertet: Niederösterreich (n=31), Oberösterreich (n=51), und Steiermark (n=16). In diesen Betrieben wurden repräsentative Proben des Grundfutters (meist AGR, aber auch Futter und TMR) sowie des Kraftfutters entnommen und durch Trocknen, Mahlen und Poolen für die Analyse vorbereitet. Die Futtermittel der einzelnen Betriebe wurden je nach Kraftfutter/Grundfutter gepoolt, und die Gehalte von 10 Phytoöstrogenen (Biochanin, Coumestrol, Daidzein, Daidzin, Formononetin, Genistein, Genistin, Glycitein, Glycitin und Ononin) der Ration mittels Flüssigchromatographie/Tandem-Massenspektrometrie (LC-MS/MS) analysiert. Um das Vorkommen und die Konzentration der identifizierten Phytoöstrogene insgesamt und in konventionellen versus ökologischen Betrieben zu beschreiben wurde eine statistische Analyse durchgeführt.

Jede der untersuchten Proben enthielt mindestens vier verschiedene Phytoöstrogene, fünf Proben wurden positiv auf alle zehn getestet. Biochanin, Daidzein und Genistein waren in allen Proben allgegenwärtig. Die Gesamtmenge der Phytoöstrogene in den Proben reichte von 4.260 µg/kg TM bis 411.420 µg/kg TM und lag im Durchschnitt bei 83.224 µg/kg TM. Zusätzlich wurden neun Biobetriebe neun vergleichbaren konventionellen Betrieben gegenübergestellt. Ausschlaggebend für die Auswahl der Betriebe waren die geografische Nähe sowie ein ähnlicher Anteil und eine ähnliche Auswahl an Futtermitteln in der Fütterung. Vier Phytoöstrogene (Coumestrol, Ononin, Daidzein, Genistein) wurden signifikant häufiger in biologischen Proben gefunden. Unter Berücksichtigung der signifikant höheren Menge an Grassilage, die in Biobetrieben verwendet wird, war nur Coumestrol signifikant höher ($P < 0,001$). Nach Vergleich mit veröffentlichten Grenzwerten stellen die ermittelten Gehalte an einzelnen Phytoöstrogenen kein ernsthaftes Risiko für die Reproduktionsleistung der jeweiligen Herden dar. Diese Werte stammen aus repräsentativen Stichproben von Futtermitteln, was bedeutet, dass einige spezifische Einzelkomponenten höhere Konzentrationen an Phytoöstrogenen aufgewiesen haben können. Die Interaktion von Phytoöstrogenen und anderen östrogenen Substanzen (z.B. Mykoöstrogene) ist ein Schwerpunkt der aktuellen Forschung und bietet noch viel Potential.

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9. Bibliography

Adams NR. 1995a. Detection of the effects of phytoestrogens on sheep and cattle. Journal of animal science, 73 (5): 1509–1515. DOI 10.2527/1995.7351509x.

Adams NR. 1995b. Organizational and activational effects of phytoestrogens on the reproductive tract of the ewe. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.), 208 (1): 87–91. DOI 10.3181/00379727-208-43837.

Adler SA, Purup S, Hansen-Møller J, Thuen E, Steinshamn H. 2015. Phytoestrogens and their metabolites in bulk-tank milk: effects of farm management and season. PloS one, 10 (5): e0127187. DOI 10.1371/journal.pone.0127187.

Adlercreutz H. 2002. Phyto-oestrogens and cancer. The Lancet. Oncology, 3 (6): 364–373. DOI 10.1016/s1470-2045(02)00777-5.

Adzersen KH, Strowitzki T. 2003. Endokrinologie der Phytoöstrogene. Gynäkologische Endokrinologie, 1 (1): B15-B25. DOI 10.1007/s10304-003-0016-7.

Aziz M, Kumar R, Ahmad N. 2003. Cancer chemoprevention by resveratrol: In vitro and in vivo studies and the underlying mechanisms (review). International journal of oncology, 23: 17–28. DOI 10.3892/ijo.23.1.17.

Benassayag C, Perrot-Applanat M, Ferre F. 2002. Phytoestrogens as modulators of steroid action in target cells. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 777 (1-2): 233–248. DOI 10.1016/s1570-0232(02)00340-9.

Bennetts HW, Underwood EJ, Shier FL. 1946. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Australian veterinary journal, 22 (1): 2–12. DOI 10.1111/j.1751-0813.1946.tb15473.x.

Bhagwat S, Haytowitz DB. 2016. USDA Database for the Flavonoid Content of Selected Foods. DOI 10.15482/USDA.ADC/1324465.

Bhattacharya A, Sood P, Citovsky V. 2010. The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. Molecular plant pathology, 11 (5): 705–719. DOI 10.1111/j.1364-3703.2010.00625.x.

Bickoff EM, Booth AN, Lyman RL, Livingston AL, Thompson CR, Deeds F. 1957. Coumestrol, a new estrogen isolated from forage crops. Science (New York, N.Y.), 126 (3280): 969–970. DOI 10.1126/science.126.3280.969-a.

Bingham SA, Atkinson C, Liggins J, Bluck L, Coward A. 1998. Phyto-oestrogens: where are we now? The British journal of nutrition, 79 (5): 393–406. DOI 10.1079/bjn19980068.

Branca F, Lorenzetti S. 2005. Health effects of phytoestrogens. Forum of nutrition, (57): 100–111. DOI 10.1159/000083773.

Burton JL, Wells M. 2002. The effect of phytoestrogens on the female genital tract. Journal of clinical pathology, 55 (6): 401–407. DOI 10.1136/jcp.55.6.401.

Daems F, Romnee J-M, Heuskin S, Froidmont É, Lognay G. 2016. Analytical methods used to quantify isoflavones in cow's milk: a review. Dairy science & technology, 96: 261–283. DOI 10.1007/s13594-015-0276-8.

D'Alessandro TL, Boersma-Maland BJ, Peterson TG, Sfakianos J, Prasain JK, Patel RP, Darley-Usmar VM, Botting NP, Barnes S. 2005. Metabolism of phytoestrogen conjugates. Methods in enzymology, 400: 316–342. DOI 10.1016/S0076-6879(05)00019-4.

Dixon R. 2004. Phytoestrogens. Annual review of plant biology, 55: 225–261. DOI 10.1146/annurev.arplant.55.031903.141729.

Gierus M, M K, Schulz H. 2012. Übertragung von Phytoöstrogenen aus Leguminosen in die Milch - Eine Betrachtung entlang der Wertschöpfungskette -. Berichte Uber Landwirtschaft -Hamburg-, 90: 354–379.

Grgic D, Varga E, Novak B, Müller A, Marko D. 2021. Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed.

Hashem N, Soltan Y. 2015. Impacts of phytoestrogens on livestock production: A review.

Hättenschwiler S, Vitousek PM. 2000. The role of polyphenols in terrestrial ecosystem nutrient cycling. Trends in ecology & evolution, 15 (6): 238–243. DOI 10.1016/s0169-5347(00)01861-9.

Herrmann KM. 1995. The Shikimate Pathway as an Entry to Aromatic Secondary Metabolism. Plant Physiology, 107 (1): 7–12. DOI 10.1104/pp.107.1.7.

Hessenberger S, Botzi K, Degrassi C, Kovalsky P, Schwab C, Schatzmayr D, Schatzmayr G, Fink-Gremmels J. 2017. Interactions between plant-derived oestrogenic substances and the mycoestrogen zearalenone in a bioassay with MCF-7 cells. Polish journal of veterinary sciences, 20 (3): 513–520. DOI 10.1515/pjvs-2017-0062.

Höjer A, Adler S, Purup S, Hansen-Møller J, Martinsson K, Steinshamn H, Gustavsson A-M. 2012. Effects of feeding dairy cows different legume-grass silages on milk

phytoestrogen concentration. Journal of dairy science, 95 (8): 4526–4540. DOI 10.3168/jds.2011-5226.

Hu J-Y, Aizawa T. 2003. Quantitative structure-activity relationships for estrogen receptor binding affinity of phenolic chemicals. Water research, 37 (6): 1213–1222. DOI 10.1016/S0043-1354(02)00378-0.

IARC. 1993. Toxins derived from Fusarium graminearum, F. culmorum and F. crookwellense: zearalenone, deoxynivalenol, nivalenol and fusarenone X. IARC monographs on the evaluation of carcinogenic risks to humans, 56: 397–444.

Ibarreta D, Daxenberger A, Meyer HH. 2001. Possible health impact of phytoestrogens and xenoestrogens in food. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 109 (3): 161–184. DOI 10.1034/j.1600-0463.2001.090301.x.

Kallela K, Heinonen K, Saloniemi H. 1984. Plant oestrogens; the cause of decreased fertility in cows. A case report. Nordisk veterinaermedicin, 36 (3-4): 124–129.

Kelly GE, Nelson C, Waring MA, Joannou GE, Reeder AY. 1993. Metabolites of dietary (soya) isoflavones in human urine. Clinica chimica acta; international journal of clinical chemistry, 223 (1-2): 9–22. DOI 10.1016/0009-8981(93)90058-c.

Kowalczyk-Zieba I, Woclawek-Potocka I, Piskula MK, Piotrowska-Tomala KK, Boruszewska D, Bah MM, Siemieniuch MJ, Skarzynski DJ. 2011. Experimentally induced mastitis and metritis modulate soy bean derived isoflavone biotransformation in diary cows. Theriogenology, 76 (9): 1744–1755. DOI 10.1016/j.theriogenology.2011.07.010.

Křížová L, Dadáková K, Kašparovská J, Kašparovský T. 2019. Isoflavones. Molecules (Basel, Switzerland), 24 (6). DOI 10.3390/molecules24061076.

Kuiper, George G. J. M., Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-Å. 1998. Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β. Endocrinology, 139 (10): 4252–4263. DOI 10.1210/endo.139.10.6216.

Kurzer MS, Xu X. 1997. Dietary phytoestrogens. Annual review of nutrition, 17: 353–381. DOI 10.1146/annurev.nutr.17.1.353.

Lamuela-Raventos RM, Romero-Perez AI, Waterhouse AL, La Torre-Boronat MC de. 1995. Direct HPLC Analysis of cis- and trans-Resveratrol and Piceid Isomers in Spanish Red Vitis vinifera Wines. Journal of agricultural and food chemistry, 43 (2): 281–283. DOI 10.1021/jf00050a003.

Lewis NG, Davin LB. 1999. 1.25 - Lignans: Biosynthesis and Function. In: Barton D, Nakanishi K, Meth-Cohn O, eds. Comprehensive Natural Products Chemistry. Oxford: Pergamon, 639–712.

Lookhart GL. 1980. Analysis of coumestrol, a plant estrogen, in animal feeds by high-performance liquid chromatography. Journal of agricultural and food chemistry, 28 (3): 666–667. DOI 10.1021/jf60229a034.

Lundh T. 1995. Metabolism of Estrogenic Isoflavones in Domestic Animals. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.), 208 (1): 33–39. DOI 10.3181/00379727-208-43828.

Mattivi F, Reniero F, Korhammer S. 1995. Isolation, Characterization, and Evolution in Red Wine Vinification of Resveratrol Monomers. Journal of agricultural and food chemistry, 43 (7): 1820–1823. DOI 10.1021/jf00055a013.

Mazur W, Adlercreutz HC. 1998. Naturally occurring oestrogens in food. Pure and Applied Chemistry, 70: 1759–1776.

Mazur WM, Duke JA, Wähälä K, Rasku S, Adlercreutz H. 1998. Isoflavonoids and Lignans in Legumes: Nutritional and Health Aspects in Humans 11The method development and synthesis of the standards and deuterium-labelled compounds was supported by National Institutes of Health Grants No. 1 R01 CA56289-01 and No. 2 R01 CA56289-04, and analytical work by the EU research contract FAIR-CT95-0894. The Journal of Nutritional Biochemistry, 9 (4): 193–200. DOI 10.1016/S0955-2863(97)00184-8.

Montes-Grajales D, Martínez-Romero E, Olivero-Verbel J. 2018. Phytoestrogens and mycoestrogens interacting with breast cancer proteins. Steroids, 134: 9–15. DOI 10.1016/j.steroids.2018.03.010.

Moss GP, Smith PAS, Tavernier D. 1995. Glossary of class names of organic compounds and reactivity intermediates based on structure (IUPAC Recommendations 1995). Pure and Applied Chemistry, 67 (8-9): 1307–1375. DOI 10.1351/pac199567081307.

Mostrom M, Evans T. 2011. Chapter 52 - Phytoestrogens. In: Gupta RC, ed. Reproductive and Developmental Toxicology. San Diego: Academic Press, 707–722.

Mostrom M, Evans TJ. 2018. Chapter 60 Phytoestrogens. In: . Veterinary Toxicology. : 817–833.

Mustonen EA, Tuori M, Saastamoinen I, Taponen J, Wähälä K, Saloniemi H, Vanhatalo A. 2009. Equol in milk of dairy cows is derived from forage legumes such as red clover.

The British journal of nutrition, 102 (11): 1552–1556.
DOI 10.1017/S0007114509990857.

Nielsen ILF, Williamson G. 2007. Review of the factors affecting bioavailability of soy isoflavones in humans. Nutrition and cancer, 57 (1): 1–10.
DOI 10.1080/01635580701267677.

Nikov GN, Hopkins NE, Boue S, Alworth WL. 2000. Interactions of Dietary Estrogens with Human Estrogen Receptors and the Effect on Estrogen Receptor-Estrogen Response Element Complex Formation. Environmental Health Perspectives, 108: 867.
https://link.gale.com/apps/doc/A67183905/AONE?u=anon~96ab83a9&sid=googleScholar&xid=5a6e4c30.

Njåstad KM, Adler SA, Hansen-Møller J, Thuen E, Gustavsson A-M, Steinshamn H. 2014. Gastrointestinal metabolism of phytoestrogens in lactating dairy cows fed silages with different botanical composition. Journal of dairy science, 97 (12): 7735–7750.
DOI 10.3168/jds.2014-8208.

Ososki AL, Kennelly EJ. 2003. Phytoestrogens: a review of the present state of research. Phytotherapy research : PTR, 17 (8): 845–869. DOI 10.1002/ptr.1364.

Patisaul HB, Jefferson W. 2010. The pros and cons of phytoestrogens. Frontiers in neuroendocrinology, 31 (4): 400–419. DOI 10.1016/j.yfrne.2010.03.003.

Pelissero C, Bennetau B, Babin P, Le Menn F, Dunogues J. 1991. The estrogenic activity of certain phytoestrogens in the siberian sturgeon Acipenser baeri. The Journal of Steroid Biochemistry and Molecular Biology, 38 (3): 293–299. DOI 10.1016/0960-0760(91)90100-J.

Penagos-Tabares F, Khiaosa-ard R, Nagl V, Faas J, Jenkins T, Sulyok M, Zebeli Q. 2021. Mycotoxins, Phytoestrogens and Other Secondary Metabolites in Austrian Pastures: Occurrences, Contamination Levels and Implications of Geo-Climatic Factors.

Penagos-Tabares F, Khiaosa-ard R, Schmidt M, Bartl E-M, Kehrer J, Nagl V, Faas J, Sulyok M, Krska R, Zebeli Q. 2022. Cocktails of Mycotoxins, Phytoestrogens, and Other Secondary Metabolites in Diets of Dairy Cows in Austria: Inferences from Diet Composition and Geo-Climatic Factors. Toxins, 14 (7). DOI 10.3390/toxins14070493.

Penagos-Tabares F, Sulyok M, Faas J, Krska R, Khiaosa-ard R, Zebeli Q. 2023. Residues of pesticides and veterinary drugs in diets of dairy cattle from conventional and organic

farms in Austria. Environmental pollution (Barking, Essex : 1987), 316 (Pt 2): 120626. DOI 10.1016/j.envpol.2022.120626.

Penagos-Tabares F, Sulyok M, Nagl V, Faas J, Krska R, Khiaosa-ard R, Zebeli Q. 2022. Mixtures of mycotoxins, phytoestrogens and pesticides co-occurring in wet spent brewery grains (BSG) intended for dairy cattle feeding in Austria. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 39 (11): 1855–1877. DOI 10.1080/19440049.2022.2121430.

Pettersson K, Gustafsson JA. 2001. Role of estrogen receptor beta in estrogen action. Annual review of physiology, 63: 165–192. DOI 10.1146/annurev.physiol.63.1.165.

Piller RV. 2006. Phytoöstrogene in der Ernährung und ihr Einfluss auf das Risiko für Brustkrebs [Dissertation]. Weihenstephan: Technische Universität München.

Price KR, Fenwick GR. 1985. Naturally occurring oestrogens in foods--a review. Food additives and contaminants, 2 (2): 73–106. DOI 10.1080/02652038509373531.

Reed KFM. 2016. Fertility of Herbivores Consuming Phytoestrogen-containing Medicago and Trifolium Species.

Salom JB, Castelló-Ruiz M, Pérez-Asensio FJ, Burguete MC, Torregrosa G, Alborch E. 2007. Acute effects of three isoflavone class phytoestrogens and a mycoestrogen on cerebral microcirculation. Phytomedicine : international journal of phytotherapy and phytopharmacology, 14 (7-8): 556–562. DOI 10.1016/j.phymed.2006.12.017.

Seguin P, Zheng W, Souleimanov A. 2004. Alfalfa Phytoestrogen Content: Impact of Plant Maturity and Herbage Components. Journal of Agronomy and Crop Science, 190 (3).

Setchell KDR, Clerici C. 2010a. Equol: history, chemistry, and formation. The Journal of nutrition, 140 (7): 1355S-62S. DOI 10.3945/jn.109.119776.

Setchell KDR, Clerici C. 2010b. Equol: pharmacokinetics and biological actions. The Journal of nutrition, 140 (7): 1363S-8S. DOI 10.3945/jn.109.119784.

Song TT, Hendrich S, Murphy PA. 1999. Estrogenic activity of glycitein, a soy isoflavone. Journal of agricultural and food chemistry, 47 (4): 1607–1610. DOI 10.1021/jf981054j.

Steiner D, Sulyok M, Malachová A, Mueller A, Krska R. 2020. Realizing the simultaneous liquid chromatography-tandem mass spectrometry based quantification of 1200 biotoxins, pesticides and veterinary drugs in complex feed. Journal of chromatography. A, 1629: 461502. DOI 10.1016/j.chroma.2020.461502.

Steinshamn H, Purup S, Thuen E, Hansen-Møller J. 2008. Effects of clover-grass silages and concentrate supplementation on the content of phytoestrogens in dairy cow milk. Journal of dairy science, 91 (7): 2715–2725. DOI 10.3168/jds.2007-0857.

Sulyok M, Stadler D, Steiner D, Krska R. 2020. Validation of an LC-MS/MS-based dilute-and-shoot approach for the quantification of 500 mycotoxins and other secondary metabolites in food crops: challenges and solutions. Analytical and bioanalytical chemistry, 412 (11): 2607–2620. DOI 10.1007/s00216-020-02489-9.

Tarkowská D. 2019. Plants are Capable of Synthesizing Animal Steroid Hormones. Molecules (Basel, Switzerland), 24 (14). DOI 10.3390/molecules24142585.

Turner JV, Agatonovic-Kustrin S, Glass BD. 2007. Molecular aspects of phytoestrogen selective binding at estrogen receptors. Journal of pharmaceutical sciences, 96 (8): 1879–1885. DOI 10.1002/jps.20987.

Vejdovszky K, Hahn K, Warth B, Marko D. 2016. Co-occurring mycoestrogens formed by {Fusarium} and {Alternaria species} mediate synergistic estrogenic effects. Toxicology Letters, 258: S55. DOI 10.1016/j.toxlet.2016.06.1293.

Wang L-Q. 2002. Mammalian phytoestrogens: enterodiol and enterolactone. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 777 (1-2): 289–309. DOI 10.1016/s1570-0232(02)00281-7.

Whitten PL, Naftolin F. 1998. Reproductive actions of phytoestrogens. Bailliere's clinical endocrinology and metabolism, 12 (4): 667–690. DOI 10.1016/s0950-351x(98)80010-4.

Woclawek-Potocka I, Bah MM, Korzekwa A, Piskula MK, Wiczkowski W, Depta A, Skarzynski DJ. 2005. Soybean-derived phytoestrogens regulate prostaglandin secretion in endometrium during cattle estrous cycle and early pregnancy. Experimental biology and medicine (Maywood, N.J.), 230 (3): 189–199. DOI 10.1177/153537020523000305.

Woclawek-Potocka I, Korzekwa A, Skarzynski D. 2008. Can phytoestrogens pose a danger in the reproduction of cows? Medycyna Weterynaryjna, 64: 515–519.

Yildiz F. 2006. Phytoestrogens in functional foods. Boca Raton: CRC Press.

10. List of figures

11. List of tables

12. Annex

Table 5. Performance values of LC-MS/MS analysis (n=98)

Metabolite	Apparent recovery (%)	LOD (µg/kg)	LOQ (µg/kg)
Biochanin	47	50	150
Coumestrol	100	5	15
Daidzein	42	50	180
Daidzin	43	50	180
Formononetin	39	50	150
Genistein	62	28	92
Genistin	34	66	220
Glycitein	59	31	105
Glycitin	57	25	82
Ononin	53	28	92

Table 6. List of detected phytoestrogens in samples (n=98)

Metabolite	Occurrence (%)[1]	Concentrations (µg/kg)[2]		
		Average ± SD	Median	Range
Biochanin	100	24,706 ± 16,035	25,464	1,101 - 52,051
Coumestrol	88	645 ± 1,280	153	19 - 8,286
Daidzein	100	9,115 ± 7,768	7,133	280 - 45,905
Daidzin	80	4,659 ± 4,137	3,769	189 - 23,945
Formononetin	21	114,017 ± 79,250	89,313	14,609 - 289,149
Genistein	100	13,402 ± 10,522	11,662	809 - 52,559
Genistin	85	6,814 ± 6,369	5,181	220 - 36,467
Glycitein	10	4,363 ± 3,726	3,211	381 - 14,069
Glycitin	74	930 ± 733	824	91 - 3,733
Ononin	52	742 ± 1,647	271	96 - 11,544
Phytoestrogens	100	83,224 ± 79,916	60,742	4,260 - 411,420

[1]n= 98 samples, samples with values > LOD

[2]Excluding data < LOD. In case values > LOD and < LOQ, LOQ/2 was used