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*Department of Agricultural, Food and Environmental Sciences
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Second level degree in *"Food Biosafety and Quality"*

"Content of Selected Phytoestrogens and Phenolics in Czech Alfalfa Cultivars"

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"Un vero scienziato è sempre disposto a cambiare le proprie idee e, perché no, i propri preconcetti, se questi vengono contraddetti dai fatti. Fatti accertati più e più volte in modo indipendente. Se invece sei un attivista ideologizzato preferisci metterti gli occhialini e scartare i fastidiosi fatti piuttosto che dire "mi sono sbagliato". Si tratta di onestà intellettuale, che a troppi manca."

Bressanini Dario

Sommario

La sempre maggiore attenzione verso i cibi funzionali ha portato la comunità scientifica ad approfondire lo studio sull'analisi e l'identificazione di fitestrogeni. Gli isoflavonoidi contenuti nell'erba medica (Medicago sativa L.) sono fitoestrogeni in grado di legare il recettore ERβ, attraverso il quale viene manipolato l'equilibrio ormonale negli organismi animali. Tali molecole vengono quindi cosiderate composti "nutraceutici". Questo studio si pone l'obiettivo di identificare e quantificare alcuni fitoestrogeni ed altri composti fenolici presenti in 15 cultivar di erba medica provenienti dalla Repubblica Ceca, presenti in piante coltivate in 3 campi sperimentali. A tale scopo è stato sviluppato un metodo HPLC-DAD a fase inversa che sfrutta un gradiente isocratico per la separazione di tali composti. Il metodo è stato utilizzato per la quantificazione di due composti caratteristici dei campioni, l'apigenina ed il cumestrolo, inoltre l'analisi dei cromatogrammi ottenuti ha condotto alla quantificazione di 11 picchi. I dati risultanti dalla quantificazione sono stati utilizzati per l'analisi statisctica condotta mediante l'analisi delle componenti principali (PCA) e l'analisi della ridondanza (RDA). Un' analisi con cromatografia liquida accoppiata ad un rivelatore di tipo spettrofotometrico, condotta con un metodo indipendente, è stata utilizzata al fine di confermare i risultati ottenuti e provvedere all'identificazione anche di altri composti fenolici presenti un un campione rappresentativo.

Dai risultati ottenuti, la cultivar Morava è stata identificata come quella con il più alto contenuto in cumestrolo seguita delle varietà Zuzana e Litava, mentre Jarka è stata identificata come quella con il contentuto minore. In generale il contenuto di cumestrolo variava tra 15.5 µg/g.p.s e 52.2 µg/g.p.s. Il contenuto di apigenina è risultato essere circa doppio di quello di cumestrolo e variava tra 42.8 µg/g.p.s. nella cultivar ZE XLII e 94.0 µg/g.p.s in Holyna, Morava and Zuzana. La varietà Zuzana è stata identificata come quella con il contenuto maggiore in entrambi i composti. Le analisi statistiche di correlazione non hanno individuato nessuna relazione significatica né tra il conteuto di apigenina e cumestrolo né tra il contenuto di cumestrolo e le caratteritiche delle piante analizzate quali la densistà di rami per per metro quadro e il peso medio di un singolo ramo.

Tali risultati possono contribuire sia a migliorare le conoscenze di base sulla relazione che intercorre fra background genetico e condizioni ambientali e contenuto di fitoestrogeni nell'erba medica, sia ad individuare quali varietà di erba medica possono essere più adatte per fornire maggiori quantità di fitoestrogeni.

Abstract

In functional food field, the research of nutraceutical compounds plays a very important role. Binding to estrogen receptor ERβ in humans and animals, alfalfa isoflavonoids act similarly to endogenous hormone estrogen and consequently influence the estrogen-related diseases. In this study we identified isoflavonoids phytoestrogens and phenolics in 15 Czech cultivars grown in three experimental spots and characterise accompanying compounds.

A reverse-phase gradient High Performance Liquid Chromatography (HPLC) coupled with a Diode Array Detector (DAD) method has been developed in order to quantify coumestrol and apigenin as the largest phenolic constituents. The chromatograms obtained from this method were also used for a simple profiling study using the 11 major chromatographic peaks appearing on the chromatogram. Moreover, a liquid chromatography-mass spectrometry (LC-MS) experiment provided an insight into the phenolic profile of one typical sample. Stem features, such as density and weight were further recorded and used in statistical correlation analysis. Principal component analysis (PCA) and redundancy analysis (RDA) were performed to compare the peaks of the DAD chromatogram.

Morava was found as the variety with the highest content of coumestrol, the highest ERβ specificity, followed by Zuzana and Litava, while the variety Jarka showed lowest content in dry mass, ranging from 15.5 µg/g to 52.2 µg/g. The apigenin content was approximately double that of coumestrol. It was found in mean concentrations ranging from 42.8 µg/g in ZE XLII to 94.0 µg/g in surprisingly all Holyna, Morava and Zuzana cultivars. Thus, Morava and Zuzana are the varieties with the highest concentration of both the compounds. No correlation between apigenin and coumestrol content was found neither between both the compounds and stem features. No specific and significant patterns were found by the PCA and RDA analysis.

We believe that these results provide partial insight into how plants and their phytoestrogenic contents are influenced by the environmental conditions and genetic background of the cultivars.

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List of Abbreviation

4CL 4-Coumarate-CoA ligase
AW Average Stems Weight
CHS Chalcone Synthase
CT Condensed Tannins
DAD Diode Array Detector
DMY Dry Matter Yield
ERβ Estrogen Receptor β
HCl Hydrochloric Acid
HDL High Density Lipoprotein
HPLC High Performance Liquid Chromatography
IFS Isoflavone Synthase
LC Liquid Chromatography
LDL Low Density Lipoprotein
LOD Limit Of Detection
LOQ Limit Of Quantification
MS Mass Spectrometry
MSL Maximum Stem Length
NADPH Nicotinamide Adenine Dinucleotide Phosphate
PAL Phenilalanin ammonia lyase
PCA Principal Component Analysis
PFP Pentfluorophenyl
RDA Rendundancy Analysis
RR Roundup Ready
Rs Resolution
RT Retention Time
SD Stem Density
UDP Uridine Diphosphate Glucose
UK United Kingdom
UPLC Ultra Pressure Liquid Chromatography
US United States
USA United States of America
UV Ultraviolet
UV-B Ultraviolet B

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1 Introduction

Alfalfa (*Medicago sativa* L.) is perennial herbaceous species belonging to the family of the Fabaceae and it is also known with the name of Lucerne especially in UK. Alfalfa is native of temperate climate such as the north of Africa where it is thought to have originated. Nowadays, it is one of the most important crop in the world because of its use as a livestock fodder especially in the North America and in Europe (Faostat, 2014). It is believed that alfalfa evolved in an area with a strong continental climate with cold winters, short, hot dry summers and late springs with soils that are near neutral in pH, well drained, and subsoil with a high lime content (Marble, 1989). This general area would include the modern political division of Turkey, Syria, Iran, Iraq, west Pakistan and Kashmir. Alfalfa spread to northern Europe in the 15-17[th]centuries, and into Russia during the 18th century. Its successful introduction to north America in the 19th century was the beginning of a rapid expansion and improvement of alfalfa (Marble, 1989).

Alfalfa is a key part of western agriculture, and has been for long time. It is cultivated in more than 80 countries. Among them, USA is the major producer with 73% of the total amount of this forage (Faostat, 2014). World production of alfalfa was around 306 million tons in 2012. There was a drop in the world production from more than 400 tonnes in recent years. In the Czech Republic, a drop in the production might be seen during last 20 years from 4.8 million tons in 1993 to 1.8 million tons in 2012 (Faostat, 2014)

USA played a fundamental role in alfalfa development, indeed it is nation's' 4th largest commodity after corn, wheat and soybean. Nowadays, alfalfa is a critical rotation crop and cash crop in all western states. Alfalfa is

also important due to its high biomass production. The record yield of one acre of alfalfa is 10 tons without irrigation and 24 tons/acre with irrigation (Breuer *et al.*, 2001).

Alfalfa is a widely adapted crop, energy-efficient and an important source of biological nitrogen fixation. Thus, it helps in reducing the need to apply expensive nitrogen fertilizers. In all parts of the word, alfalfa is recognized as having the capability of providing from 100-200 kg/ha of nitrogen for use by subsequent cash crops such cotton, corn and wheat (Putnam *et al.*, 2000).

Moreover, alfalfa is directly consumed by humans in the form of alfalfa sprouts. According to the International Sprout Growers there was approximately $250 million dollars worth of sprouts sold in North America in the 2000. Alfalfa juice is also used in some health food products (Breuer *et al* ., 2001).

In addition to the traditional uses of alfalfa as an animal feed, it is beginning to be used as a bio-fuel for the production of electricity, bioremediation of soils with high levels of nitrogen, and as a factory for the production of industrial enzymes such as lignin peroxidase, alpha-amylase, cellulase, and phytase (North American Alfalfa Improvement conference, 2014).

Since 2011 GMO Alfalfa is allowed by the USDA to be growth in USA. The GMO Alfalfa is commercialized by Monsanto Company. Planting started in the USA in 2005 but was regulated in 2007 when a District Court found that risks of cross-contamination with non-GM alfalfa had not been sufficiently assessed by the USDA. The ban was reversed in June 2010 by the US Supreme Court and planting resumed in January 2011. This plant is called Roundup Ready Alfalfa and it was developed through the insertion of a bacterial gene that confers resistance to glyphosate, a common herbicide also known as Roundup. Growers can spray their fields with the glyphosate herbicide and all the grass will be killed by it except for the RR Alfalfa (Gilla, 2011).

2 Aim of the thesis

The aim of our research is to clarify the variation of phenolic compounds in alfalfa. These compounds are known to vary among different conditions of environmental stress, but are also differentially produced among varieties of the plant. Currently, the knowledge on how varieties differ in phytoestrogens contents is limited.

The aim of this study was thus to investigate the phenolics and in particular phytoestrogens content in Czech varieties of alfalfa with a special focus on two major phenolic compounds: coumestrol and apigenin. The high performance liquid chromatographic (HPLC) method was thus developed. Besides, we conducted a comprehensive principal component analysis (PCA) and redundancy analysis (RDA) statistical analysis based on the HPLC chromatogram profile to get an insight into differences in other yet unknown phenolics content between the varieties. The peak identity was further confirmed by the liquid chromatography-mass spectrometry (LC-MS) and by means of their behaviour after hydrolysis.

This study is a part of a larger experimental design on isoflavonoids content of alfalfa varieties under different agricultural management. Knowledge on flavonoids and isoflavonoids content and their variations among varieties might be of interest for cosmetic, food or pharmaceutical industry.

3 Review of the state of the art

3.1 Alfalfa as fodder

Alfalfa is usually cultivated for hay, and is frequently used for silage or haylage, dehydrated to make meal or pellets, or used fresh by grazing or cut-and-carry.

Alfalfa hay is used primarily as animal feed for dairy cows but also for horses, beef cattle, sheep, chickens, turkeys and other farm animals and it is universally considered one of the most highest-quality forage. Alfalfa has also been historically recognized for its medical value for sick animals because its beneficial effect are well known since a long time ago (Vassart and Greth, 1991). One of the most important features of this fodder is its high nutritional quality because it contains between 15 to 22% of crude protein as well an excellent source of vitamins and minerals (Radovic, 2009). This characteristic leads alfalfa to become perfectly suitable for the lactating dairy cows. Cows can consume a large quantities of alfalfa and produce more milk when alfalfa silage is fed as the sole source of forage.(Nelson and Satter, 1990). The high forage protein content of alfalfa meet the need of the market, particularly since concentrates of animal origin were banned in the European Union (Veronesi *et al.*, 2011)

The importance of alfalfa in the agronomical world is due to its properties as nitrogen source for other rotational crops, thus also as soil improving crop, and in the livestock field as complete source of nutrients for the production of meat and milk (Tracy and Faulkner, 2006);

Although alfalfa is well known also as the "Queen of fodder" (Ahmed *et al.*, 2013) there are some disadvantages that must be considered. Alfalfa has high concentration of phenolic compounds which are probably useful in

protection of the plant against the attack by microorganism or insect. The same compounds which provide the plants defence may be either dangerous or healthy when they are eaten by human or animals. These compounds are basically secondary metabolites and their features and behaviour is well explained in the following chapters. Despite all the positive properties mentioned, alfalfa has some risks related to high intake which involve for example bloating in goat or infertility in sheep (Braden *et al.*, 1967). Previous studies showed that cows fed estrogenic forage may suffer impaired ovarian function, often accompanied by reduced conception rates and increased embryonic loss in female while males are relatively unaffected. Although this kind of infertility is temporary, the cattle exposed to phytoestrogens for prolonged periods may suffer a second form of infertility which is permanent (Adams, 1995). However, phytoestrogens, which are dangerous for animal, represent the most interesting constituents in the human nutrition fiend because of their nutraceutical effects in the human organism.

3.2 Secondary metabolites in Alfalfa

Plants produce a great diversity of low molecular weight compounds. These can be divided into two main classes, the primary metabolites and the secondary metabolites. By the term "primary metabolites" means those compounds which are involved in the fundamental processes for the plant world metabolism. This class include only a minor rate of all the metabolites. The other compounds are termed secondary metabolites and they are defined as compounds whose biosynthesis is restricted to a selected plant group (Pichersky and Gang, 2000). The restricted distribution of many such compounds enables them to be used as taxonomic markers in most of the cases. Moreover, they provide the major contribution to the specific odours, taste and colour of plants. Secondary metabolites pathways have an evolutional reason to exist because they lead to many abilities proper of the plant world. For

instance, the ability to ward off pathogens and herbivores or suppress the growth of neighbouring plant is due to the capability to synthesize toxic chemicals which are secondary metabolites (Bennett and Wallsgrove, 1994).

The secondary metabolites class includes a wide diversity of compounds characterized by different chemical structures and properties, systemized into terpenoids and other isoprenoids, flavonoids and phenolic compounds, nitrogen-containing alkaloids and sulphur-containing compounds (Crozier *et al.*, 2006).

Alfalfa plants are rich in secondary metabolites belonging to several classes which are extensively presented in the following chapters. Particular attention will be given to the class of flavonoids which are mostly involved in the nutraceutical field.

3.2.1 Saponins

The saponins are a class of molecules that have an antimicrobial and haemolytic ability. They are amphipathic glycoside of lipophilic triterpene derivative. Saponin content in alfalfa forage may have adverse haemolytic effects on livestock and reduce growth and egg-production in poultry (Munro, 2009). Anti-herbivorous haemolytic saponins are present in high concentrations in certain alfalfa lineages and cultivars.

Some saponins show allelopathic, antimicrobial, and anti-insect activity, but they can also be toxic to monogastric animal, or they can be a factor of antipalatability and they can reduce the forage digestibility in ruminants.

A study by Klita *et al.* 1996 show that alfalfa saponins have a bad effect in the animal health causing bloat in sheep and in cattle; this effect is partially due to the inhibition of the rumen motility and the reduction of the microbial nitrogen outflow. Saponins, when administered pure in the concentration of 4% of the total weight of fodder, eliminate ruminal contraction within minutes and suppressed activity for several hours (Klita *et al.*, 1996).

Monogastric animals often avoid consuming foods that contain saponins. Therefore, development of saponins free alfalfa is an agronomic target. On the other hand saponins have also some pharmacological activities such as the anticholesterolemic properties (Dixon and Sumner, 2003).

In humans, the there are no data on the effect of saponins from clinical studies. These compounds are not so relevant because at the moment the consumption of alfalfa in human is not so common and the varieties used for this purpose are characterized by a low content of these compounds. These compounds are particularly interesting for the food factory fields due to their antimicrobial ability that can be used to increase the conservation period of food avoiding the use of synthetic chemical compounds (Tajkarimi *et al.*, 2010).

3.2.2 Condensed tannins

The bloating effect of the alfalfa saponins is hindered by another class of molecules which reduce the disadvantages: the condensed tannins (CT) (Jensen *et al.*, 2013). Condensed tannins (proanthocyanidins, polyflavonoid tannins, catechol-type tannins, pyrocatecollic type tannins, non-hydrolyzable tannins or flavolans) are polymers formed by the condensation of flavans. They do not contain sugar residues. They are called proanthocyanidins as they yield anthocyanidine when depolymerised under oxidative conditions.

This class of compounds plays a protective role within the plant, but is now attracting attention because of its vast effects on human health and immunity. This effect is due to the CT and also by their precursors catechins and epicatechins. CTs bind to dietary proteins in the rumen thereby they slow down their rate of degradation, preventing bloat and improving the animal's nitrogen nutrition by increasing the amount of dietary protein exiting the rumen. This can lead to increased body weight and wool production. In addition to reducing bacterial degradation of proteins in the rumen, CTs can also slow down protein

degradation during ensiling of forage legumes thereby improving the nitrogen nutritional value of the feed (Dixon and Sumner, 2003).

New research is looking for a way to create, by the genetic engineering, a new alfalfa species with a higher CTs accumulation for the protection of animals against pasture bloat (Kumar, 2011). These compounds are also important as part the human diet. They are one of the reasons why tea as is considered as a healthy beverage since a long time ago (Lin *et al.*, 2014). They have many important features that are considered beneficial for human health such as strong antioxidant ability due to the phenolic moieties, that include the possibility scavenge free radicals and the reduction of the peroxidation of lipids, they also have an anti mutagen activity that can help in prevention of cancer development (Chung *et al.*, 1998).

3.2.3 Flavonoids

Flavonoids are secondary metabolites widely distributed in the plants world which origin from the phenylalanine and the malonyl-CoA.so they represent the connection between two of the most important biosynthetic pathways of plants, the shikimate pathway and the fatty acid pathway (Dewick, 2011).

Flavonoids play several important roles in the vegetal organism especially acting in plant defence against pathogens such as fungi or microorganism and from the toxicity of radical molecules that can be dangerous (Baetz and Martinoia, 2014). Flavonoids have a good antioxidant capacity due to their hydroxyl groups associated to the benzene rings that gives to flavonoids a low redox potential and capacity to quench the radicals (Wenli *et al.*, 2004). Furthermore they are able to absorb the UV radiation and for this reason they are concentrated in the leaf's surface in order to protect the plant from the damage that that radiation which can cause to the DNA structure (Zavala *et al.*, 2014). It is also thought that they play a role in the symbiosis between the plant and the nitrogen fixing microorganism (Mapope and Dakora, 2013).

Flavonoids are also produced in the subterranean part of the Leguminosae species as root exudates and for this reason they are thought to be involved in the symbiosis between the root and microorganisms which fix atmospheric nitrogen (Baetz and Martinoia, 2014). A study of Hassan and Mathesius shows how these compounds can influence the formation of the rhizobium nodules (Hassan and Mathesius, 2012). According to their structure, flavonoids have been shown to stimulate or inhibit rhizobial *nod* gene expression, improving chemo attraction of rhizobia towards the root, inhibition of root pathogens, stimulating mycorrhizal spore germination and hyphal branching (Hassan and Mathesius, 2012).

3.3 Biosynthetic origin of flavonoids

Flavonoids and isoflavonoids are synthesized by a branch of the phenypropanoid pathway. This extended metabolic route is also involved in the synthesis of other important plant compounds such as tannins, lignins, lignans, anthocyanins, flavones, flavonols and other (Dewick, 2011). The precursor of the pathway is the amino acid L-phenylalanine, which, in the initial step, is stripped of its amine group to produce cinnamic acid via the enzyme phenylalanine ammonia lyase (PAL). In the second and third reaction, cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL) convert cinnamic acid into *p*-coumaryol CoA. The first critical enzyme for flavonoids synthesis is chalcone synthase (CHS), which exists as a multigene family in soybean. All flavonoids are derived from a chalcone precursor, which is the product of the condensation of 4-coumaroyl CoA (a product of the central phenylpropanoid pathway) and three molecules of malonyl CoA by the enzyme chalcone synthase (CHS). It has recently become apparent that CHS is just one member of a family of plant polyketide synthases that together form a variety of natural products (Abe and Morita, 2010). The enzymes belonging to these apparently complex pathways for the elaboration of basic flavonoid skeletons,

fall into a few biosynthetic pathways that produces some of the major classes of compounds such as flavonoids, isoflavonoids and anthocyanin. CHS is an homodimer which is made of two subunits containing two functionally independent active sites. The architecture of the catalytic pocket defines the sequence and chemistry of the multiple decarboxylation and the condensation reactions (Dixon and Steele, 1999).

Other important enzymes in the pathway for isoflavone synthesis are chalcone isomerise (CHI), which convert chalcones to flavanones, and chalcone reductase (CHR) required for daidzein and glycitein formation. The isoflavonoids form a distinct subclass of flavonoid. They are structural different from flavonoids because the shikimate-derived aromatic ring has migrated to the adjacent carbon of the heterocycle. This rearrangement process is brought by a cytochrome P-450-dependent enzyme requiring NADPH and O cofactors, which transforms the flavanones liquiritigenin or naringenin into the isoflavones daidzein or genistein respectively, via intermediate hydroxyl isoflavanones. A radical mechanism was proposed by Dewick (Dewick, 2011).

In legumes, isoflavone synthase IFS rearranges the flavonoids skeleton of the dihydroxyflavanone produced by the CHS, leading to the accumulation of a wide range of simple isoflavonoids, coumestans, pterocarpans, and isoflavans (Dixon and Paiva, 1995).

The enzyme that specifically differentiates isoflavone-production plant species from those with no isoflavone content is isoflavone synthase (IFS), and endoplasmic reticulum integral cytochrome P450 monoxygenase, which catalise a 2,3 aryl ring migration of flavanones to their corresponding isoflavones. In the soybean genome, the gene relative to IFS is present in two copies, IFS1 and IFS2, which differ by few amino acids. Both convert naringenin and liquiritigenin to genistein and daidzein, respectively (Gutierrez-Gonzalez *et al.*, 2010). This rearrangement is quite rare in nature, and isoflavonoids are almost entirely restricted to the plant family the Leguminosae so also to alfalfa.
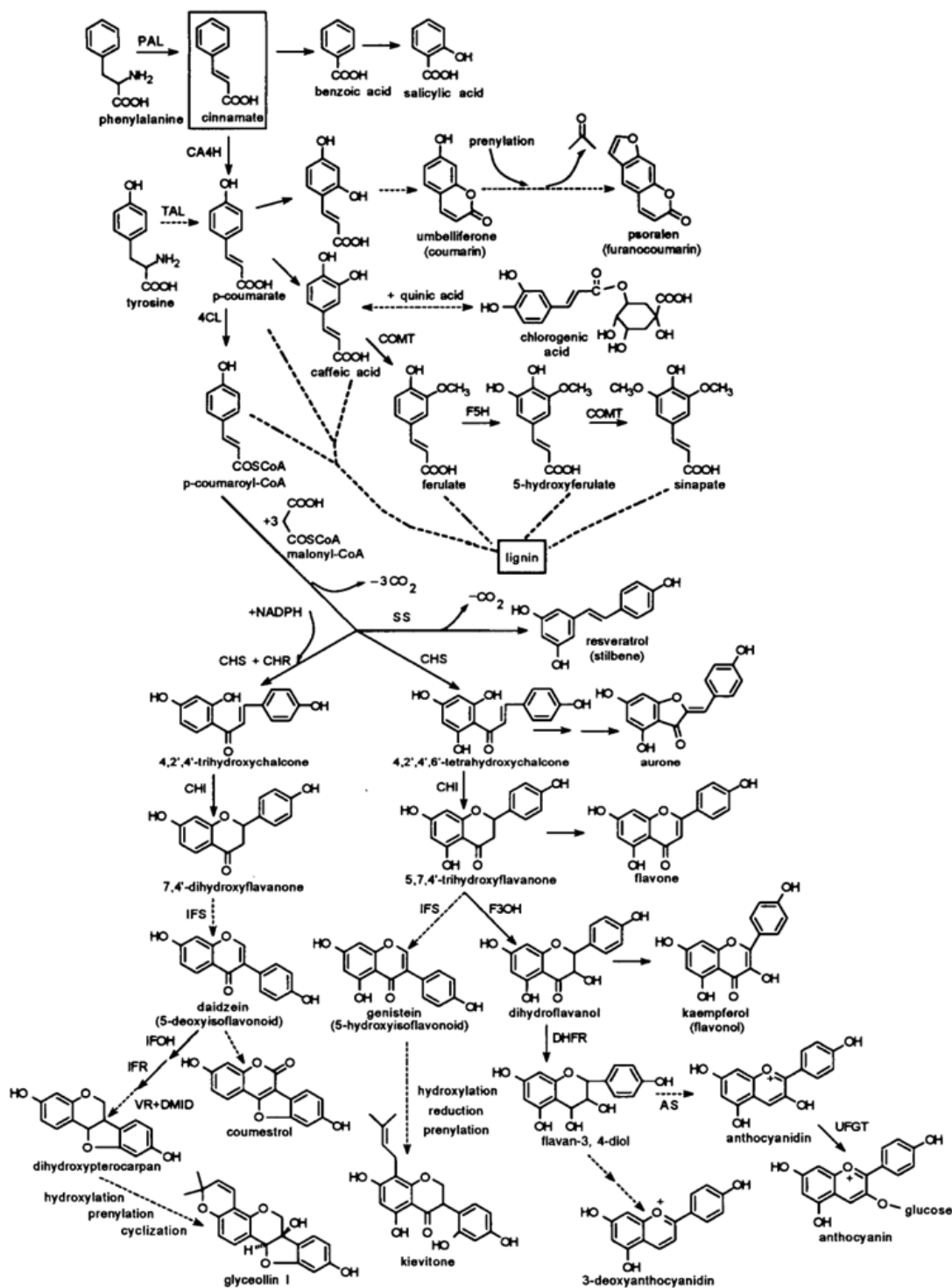
Figure 1 The biosynthesis of the major classes of flavonoids derivates.
The enzymes are: PAL Phenylalanine ammonia-lyase; CA4H Ciannamic acid 4-Hydroxylase; 4CL 4-cumarate CoA Ligase; COMT Catechol-O-methyl transeferase; F5H ferulate 5-hydroxylase;SS Stilbene Syntase; CHS, chalcone synthase; CHR, chalcone reductase; CHI,chalcone isomerase; IFS Isoflavone synthase I; F3OH,flavanone 3 hydroxylase; IFR Isoflavone Reductase (Dixon and Paiva, 1995).

Coumestans are the oxidation product of pterocarpans and their biosynthesis pathway is not clear yet. The biological activity exhibited by these compounds is of definite interest to chemist and pharmacologist (Tuskaev, 2013).

3.4 Factors influencing synthesis of flavonoids

The secondary metabolites biosynthesis is enhanced by various stress factors. The phenylpropanoid pathway is significantly influenced by biotic and abiotic stress.

Most flavonoids are antimicrobial compounds synthesized in response to pathogen attack. They belong to different classes such as pterocarpans, isoflavans, prenylated isoflavonoids, stilbenes, psoralens, coumarins, flavonols and aurnes. The concentrations of these compounds are varying in various parts of the plant, even differing between shady and sunny part of the leaf (Rasmussen *et al.*, 1991).
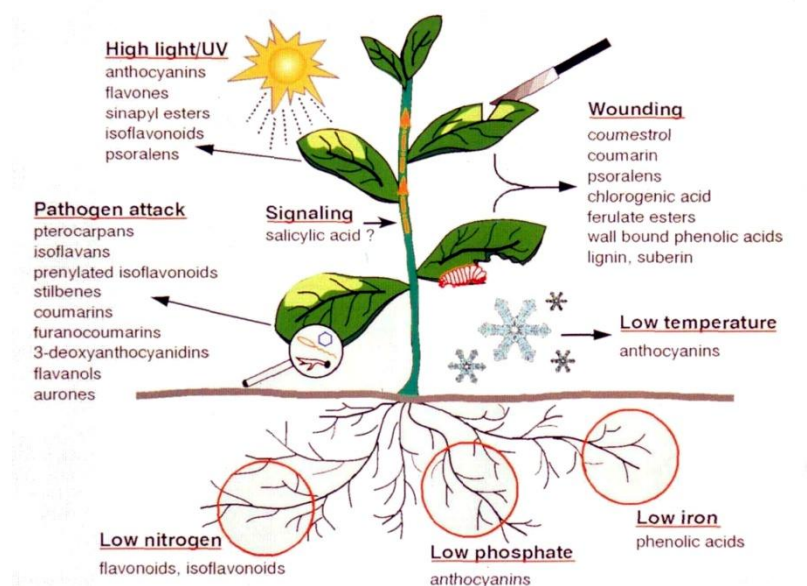
Figure 2 Example of Stress-Induced Phenylpropanoids (Dixon and Paiva, 1995).

Nutritional stresses cause increases in the concentration of phenylpropanoids in roots or root exudates (Hassan and Mathesius, 2012); for

example, low nitrogen soil content induces flavonoids and isoflavonoids *nod* gene in inducers and chemoattractans for nitrogen-fixing symbionts, whereas low iron levels can cause increased release of phenolic acids, presumably to help to solubilise metals and thereby their uptake (Graham, 1991).

Flavonoids and other phenylpropanoids have long been thought to play a role in protecting against UV radiation, because they accumulate primarily in the epidermal and hypodermal layers of the leaves and stems (the most illuminated layers) and strongly absorbs light in the UV-B wavelengths (Dixon and Paiva, 1995).

In most biological systems, induction of phenylpropanoid synthesis under stress conditions is the result of increased transcription of genes encoding the corresponding biosynthetic enzymes(Blount *et al.*, 1992)(Blount *et al.*, 1992)(Blount *et al.*, 1992) (Blount *et al.*, 1992). During stress conditions or pathogen attack the signal elicitors or stress perception with transcription or downstream response genes remain to be defined unequivocally (Blount *et al.*, 1992).

The changes involved in the reaction to the stress agent include mostly the accumulation of secondary metabolites. For instance, leaf wounding provokes the accumulation of the phenolic compound in the upper layer of the leaves. Other phenolics are accumulated after a pathogen attack in the infection site. Similarly, UV stress induces anthocyanins and other flavonoids accumulation specifically in upper epidermal cells as it is shown in Fig.2 (Dixon and Paiva, 1995).

Use of modern techniques has elegantly demonstrated that flavonoids, CHS protein, and CHS, PAL, and 4CL transcripts all accumulate in the same epidermal cells, following UV irradiation. These studies and similar indicate that stress-induced phenylpropanoids usually accumulate in the cells in which they are synthesized (Dixon and Paiva, 1995).

The sub cellular sites of phenylpropanoid synthesis are still matter of debate. Studies from Mackenbrock *et al.* shows the possibility of a final

conjugation of these molecules during the transport in the vacuole; for example the glucosyl transferase of pterocarpan conjugate synthesis are associated with the tonoplast membrane and then they will accumulate there (Barz and Mackenbrock, 1995).

3.4.1 Fertilization effect

A study by Taie *et al.* reported the influence of different kind of fertilization on the flavonoids, isoflavonoids and other phenolics compound concentration. In this study, three different fertilization modes were compared: inorganic, organic and organic plus bioorganic fertilizer.

The study was focused mostly on the concentrations of total flavonoids and isoflavonoids in plant grown in pot in greenhouse. The results show that there are not any significant differences between organic and inorganic fertilization regarding plant growth. On the other hand, organic fertilization with a bioorganic fertilizer increases slightly the flavonoids production. The bioorganic compound is facilitated by different microorganism species such as highly efficient strains of *Bradyrhizopium japonicum*; phosphate dissolving bacteria (*Bacillus megaterium* var. *phosphaticum*), *Azospirillum* spp. and *Pseudomonas* spp (Taie *et al.*, 2008).

These microorganisms provide many advantages for plants. They are able to fix atmospheric nitrogen and provide it for the plant. Furthermore they synthesize siderophores that sequester iron from the soil and make it available for the plant (Babalola, 2010).

Biofertilizers play major role in determining the levels of phenolics, total flavonoids and isoflavonoids of soy bean seed organically grown. Adding multi-biofertilizer to 50 or 75% compost resulted in great enhancement effect on concentration of total phenolics, total flavonoids, caffeic acid, genistein, quercitin and daidzedin compared with those inorganic fertilizer or organic fertilizer (Taie *et al.*, 2008).

3.4.2 *Water stress effect on isoflavonoids genes expression*

Studies showed that accumulation of isoflavones is promoted by well-watered condition, but the molecular basis remains elusive (Gutierrez-Gonzalez *et al.*, 2010).

CHS7, CHS8 and IFS2 play a key role in the biosynthetic pathway of isoflavonoids thus they are correlated with their accumulation. It was found that the two isoflavone syntase genes in soy bean (ISF1 and ISF2) showed different patterns of expression (Gutierrez-Gonzalez *et al.*, 2010). The abundance of ISF1 transcripts was maintained at the constant rate, whereas ISF2 was down-regulated and highly correlated with isoflavones accumulation under both water deficit and well-watered conditions. This fact suggest that IFS2 was down-regulated and highly correlated with isoflavone accumulation under both water deficit and well-watered conditions, suggesting IFS2 as a main contributor to isoflavone diminution under drought (Gutierrez-Gonzalez *et al.*, 2010).

Generally a short period of water stress doesn't change the flavonoids or isoflavones accumulation, but, on the other hand, a long period of drought can modify the production of these compounds. In water stress situation, the expression and the activity of the biosynthetic pathway enzymes change and so does consequently the isoflavones production. For instance the PAL enzyme is activated under stress condition and this has been judged to be a stress responsive mechanism (Dixon and Paiva, 1995).

Gene expression profiling showed that in well-watered condition transcriptional abundance of CHS7, CHS8 and IFS2 correlated well with isoflavones content. These three genes play an important role in isoflavonoids synthesis. So there is a good correlation between IFS2 transcripts and isoflavone content also exist under water-stressed condition (Gutierrez-Gonzalez *et al.*, 2010).

3.4.3 Other factors

The crop growth is not only influenced by the agronomical management of the field but also from the field and environmental characteristics such as weather or latitude. Since the secondary metabolism confers an adaptive advantage in the plant kingdom, it can be influenced by climatic ad environmental factors. For instance, UV radiation influence both the plant growth and the secondary metabolism (Kakani *et al.*, 2003). Plants exposed to intense UV radiation are characterized by a decreased height, which is primarily due to shorter internodes rather than smaller node number (Teramura, 1983). This effect is caused by the photo-oxidative destruction of the phytohormone indol acetic acid and the increment of the ethylene production (Ros and Tevini, 1995). UV radiation leads to an adaptive mechanism for the accumulation of UV absorbing compounds. The leaf epidermal layer is known to accumulate most of the secondary metabolites, such as phenolics and flavonoids that absorb UV-B radiation and avoid the UV related damage (Kakani *et al.*, 2003). Since UV exposition of crops is influenced by both weather and latitude, a study from Yuan *et al.* could gather soybean cultivar as UV tolerant the five originated from south China (low latitude) whose growth was not influenced by the UV radiation. Cultivars originating from the north China (high latitude) were found to be high sensitive (Yuan *et al.*, 2002). This study confirmed that UV radiation, and consequently latitude, influence crop growth and secondary metabolites biosynthesis. Other factors influencing secondary metabolism such as soil texture or pH are not reported.

3.5 Functional food in human nutrition

The massive recent outbreak of non-communicable diseases such as cancer or cardiovascular diseases might be caused by nutritional and lifestyle habits. Many factors are increasing the probability to get these diseases like pollution, alcohol, smoking and wrong alimentation. This is the reason why much of the

research now concentrates on strategies focused on an improvement of population health such as the concept of functional foods. Functional food is a term used to indicate foods which go beyond the average foods in content of particular, essential and difficult-to-get nutrients or their ratios and thus provide some hypothetical health benefits. Indeed it is common to find foods enriched with vitamins, minerals or antioxidants (Salminen *et al.*, 1998).

There are many different kinds of functional foods which differ in the composition and the way of proposed action. For instance, the probiotic yogurts are supposed to help the organism with the digestion. Other foods can be enriched with vitamins which are needed for a lot of enzymatic processes. One of the most popular functional proprieties of this kind of food is the antioxidant one. The antioxidants are a class of molecules which has the ability to scavenge the reactive oxygen species which are thought to be the cause of most the degenerative diseases (Ramarathnam *et al.*, 1995). A lot of food and beverages owe their popularity to this class of compounds, for example the tea is supposed to have beneficial effect and to be the reason of the low rate of degenerative disease in the Eastern countries in which the use of this beverage is more common than in other (Inoue *et al.*, 2001).

The most famous antioxidants are vitamin C (ascorbic acid) and vitamin E (tocopherol) but now much attention is paid to another class of antioxidant molecules: the flavonoids.

The recent interest in this group of compounds is stimulating researchers to invest time and energy in their identification but also in clarifying their possible role in human metabolism. It is important to mention that the proposed use of this food does not aim to cure but rather prevent diseases. Also, their commercial potential is of interest (Sawyer *et al.*, 1985).

Generally, alfalfa is consumed by humans in form of sprouts in salads or administrated orally in form of pills for food supplements. The product is marketed as rich in vitamins, especially vitamin E, minerals and phytoestrogens (Plaza *et al.*, 2003). As fresh, alfalfa, is preferred to be

consumed in form of sprouts instead of seeds, because the sprouting process increases the nutritional properties and increases the bioavailability of vitamins, mineral and phytestrogens (Bau *et al.*, 1997).

3.5.1 *Isoflavonoids effect in animals and humans*

Isoflavonoids are a class of planar molecules which plays an important role in human dietary due to their similar shape to the steroidal mammal oestrogen hormone. For this reason they can be used to mime the function of the endogen hormones (Yuan *et al.*, 2007).
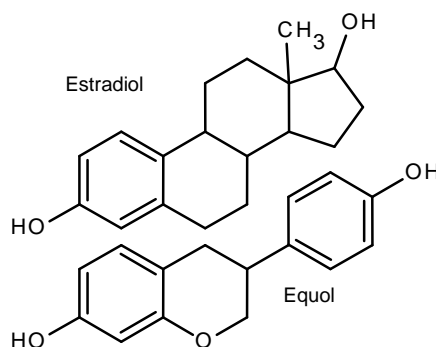
Figure 3 Comparison of the structure of isoflavone metabolite equol with that of estradiol showing the similarity in planar spatial arrangement of the two molecules.

Equol is a planar molecule which comes from the mammal metabolism of isoflavonoids. Its molecular shape characterized by the phenolic ring is a key structural element that allows equol to bind the oestrogen receptors such as the endogen oestrogen estradiol does (Setchell *et al.*, 1984). Indeed, when the structure of the isoflavones metabolite equol and estradiol are overlaid, they can be virtually superimposed as it is shown in Fig.3. The distance between the hydroxyl groups at each end of both molecules is virtually identical; this allows the binding with oestrogen receptors. For this reason they are also well known as phytoestrogens. Phytoestrogens represent just one of many important bioactive non-nutrients found in many plants commonly consumed in the human diet (Setchell and Cassidy, 1999).

Estrogens are a family of steroidal compounds that have a hormone function in animal as well in mammalian as in insect. Indeed, the sexual function of hormones has a very ancient history in the evolution process. The myriad of biological proprieties that have been associated with phytoestrogens is resulted in the current euphoria over their potential for the prevention and/or treatment of many hormone-dependent disease (Kuhnle *et al.*, 2011). Nowadays there are many epidemical studies showing the beneficial effects of phytoestrogens in areas characterized by a diet rich in isoflavones (Lee *et al.*, 2003).

There are several possible mechanisms of action of isoflavones in disease prevention, which include oestrogenic/anti-estrogenic activity, cell anti-proliferation, induction of cell cycle arrest and apoptosis, prevention of oxidation, anti-inflammatory, regulation of host immune system, and changes in cellular signalling. (Rostagno *et al.*, 2009)

Estrogens related health issues such as the osteoporosis incidence, are mostly related to pre- and post- menopausal women and isoflavones seem to influence these hormones balance binding ERβ receptors (Clarkson *et al.*, 2011).

The epidemiological studies confirm that diet rich in particular vegetables containing phytoestrogens can influence the incidence rate of oestrogen-related diseases in a population. This is hypothetically due to the intake of isoflavones acting as endogenous hormones affecting the characteristics of the menstrual cycle in pre-menopausal women by suppressing the normal midcycle surge in follicle-stimulating hormone and luteinizing hormone (Phipps *et al.*, 1993).

Other studies on animals showed tumour cells growth inhibition as results of the action of the isoflavonoids daidzein and genistein. Genistein can also influence the key transcription factors that are involved in the expression of stress response-related genes involved in programmed cell death. This ability was also shown to act on the *in vitro* breast cancer cells although the

mechanism of action is not clear yet. It is more likely that the beneficial effects of these compounds particularly *in vivo*, are the results of multiple actions (Akiyama *et al.*, 1987).

Cardiovascular diseases

Isoflavones seem to be involved also in the cardiovascular health. Estrogens deficiency is associated with significant alteration in lipoprotein metabolism and with serum cholesterol concentration, which is markedly increased in the post-menopausal age (Wuttke *et al.*, 2002). Reduction in serum cholesterol is associated with reduced risk or cardiovascular disease (Sarrel, 1990). The cardio protective effects of oestrogen replacement therapy are mediated by the effects on lipid metabolism which include lowering LDL cholesterol content and increasing the level of HDL cholesterol and also by the direct action on the blood vessel wall (Sarrel, 1990). The exact mechanism of the hypocholesterolemic effect of these compounds remains elusive and it is almost certain to be multifactorial. The main role seems to be the up-regulation of the LDL-receptor activity (Kirk *et al.*, 1998).

A linear relationship was observed between isoflavones content and blood cholesterol reduction in monkeys by Sirtori *et al.*. The hypocholesterolemic effect was lost when the isoflavones were removed from the vegetal matrix by ethanol extraction (Sirtori *et al.*, 1998).

In addition, the effect on cholesterol seems to provide other relevant benefits in reducing the risk for cardiovascular disease. For instance, genistein enhances resistance of LDL to the oxidation *in vitro* because it is the most powerful antioxidant among the isoflavones present in soy protein. Furthermore, genistein inhibits the process of blood coagulation, which is a key promoter of plaque formation. This effect is due to the inhibition of release growth factors, such as plated-derived growth factors, resulting in the inhibition of thrombin formation. These effects may be due to the genistein

inhibition of tyrosine kinase which is a central enzyme in thrombin formation and inflammation centre (Setchell and Cassidy, 1999).

Phytoestrogens and reproduction

Isoflavonoids are exogenous oestrogen-like molecules that can promote or inhibit reproductive processes (Alexander, 2014). The effects of these compounds depend on the final serum level of bioactive compound which is influenced by many factors such as route and timing of exposition (Jefferson *et al* ., 2012). Numerous epidemiological and clinical studies evaluated the relationship between soy phytoestrogens consumption and its impact on reproductive health. A study from Chen *et al.* analyzes the effect of phytoestrogens in female rats. This study shows how the exposure to purified genistein affects the ovarian development increasing the percentage of multi-oocyte follicles. Furthermore, this treatment acts also on the oestrous cyclicity, the ovarian function and the timing of vaginal opening causing the decrease of fertility and increasing the incidence of uterine adenocarcinoma (Chen *et al* ., 2007). The results of this study are relative to pure genistein given by subcutaneous injection of the aglycone which is much different than consuming food containing isoflavones because it bypasses the gut metabolism (Cederroth *et al* ., 2012).

In order to understand the effect of phytoestrogens from the male point of view, a study from Atanassova *et al* . is of importance. This study analyzed the result of subcutaneous injection of estrogens solution in rats. The evaluated rats presented a decreased spermatogenesis and a lower level of testosterone in blood (Atanassova *et al* ., 2000). However, again, these results present different conditions from the physiological conditions after oral intake of isoflavones. This is probably the reason why an epidemiological study by Chavarro *et al* . is supporting exactly the opposite result. Chavarro *et al* . study reports no significant relation between food rich in phytoestrogens consumption of and any reproduction problem or decrease fertility (Chavarro *et al* ., 2008).

This finding suggests that the effect of the injected aglycone is more active on the reproduction health than phytoestrogens intake by vegetable and implicates a potential use of the pure aglycones for pharmaceutical purpose.

Osteoporosis

Isoflavones have several different clinical effects; this chapter wants to focus on the effect against the osteoporosis.

Epidemiological studies show a lower rate of bone crusher due to bone loss in post-menopausal women in Eastern countries than in the in European women (Atmaca *et al* ., 2008). From the epidemiological results it is difficult to discern whether this difference is accounted for isoflavones intake or other factors that can influence the osteoporosis incidence (Tobias *et al* ., 1994). In spite of this, a study from Srivastava *et al.* provides evidence that phytoestrogens supplementation in animal models during the skeletal growth could enhance bone formation and afford greater bone conservation. Thus, according with this study, the supplementation of phytoestrogens may provide an effective strategy for mitigation of the developing osteoporosis and fragility fracture after menopause age (Srivastava *et al.*, 2014).

Isoflavones and their metabolites are able to bind the ERβ estrogens specific receptors placed on the bone (Barnes *et al.*, 2000). By this ability, they will influence the gene expression of enzymes involved in increases of mineral bone content preventing the bone loss due to oestrogen deficiency associated with menopause (Paterni *et al.*, 2014).

A study from Draper *et al.* showed that coumestrol, the main coumestan present in alfalfa, shows the bone conservative effect in the ovariectomized rat model when given intramuscularly (Draper *et al* ., 1997).

Phytoestrogens and cancer

Epidemiological studies confirmed that in Eastern countries such as China, Korea and Japan, the incidence of cancer can be up to five times below the

average in Western countries, and this is thought to be due to the high dietary intake of phytoestrogens (Magee and Rowland, 2012).

The carcinogenesis is a malignant transformation of healthy cells that can be associated with increase DNA mutations, cell proliferation, decreased apoptosis, immune response and other processes whose may be under control of estrogens (Alexander, 2014).

Phytoestrogens, which include lignans, coumestans, isoflavonoids and stilbenes, have the ability to regulate the expression of proteins involved in controlling cell cycle, apoptosis, and in the modulation of signal transmission from growth factors and membrane-associated estrogens receptors (Rice and Whitehead, 2014).

To confirm this theory, a study from Wang *et al.* analyzed the property of apigenin against the cancer development. Apigenin was found to inhibit the cell-cycle progression in different colon carcinoma cell lines *in vitro*. It seems to act on cell-cycle inhibiting cellular proliferation by delaying cell cycle progression at G2/M and/or G1 checkpoints depending on the specificity of cell type (Wang *et al.*, 2000).

Isoflavonoids such as genistein was found to be a powerful inhibitor of angiogenesis and metastasis. Both *in vivo* and *in vitro* studies suggest genistein as a promising reagent for cancer chemoprevention and/or treatment as well as other isoflavonoids (Alexander, 2014).

Toxicity and placebo effect

Although isoflavonoids are known for their beneficial effects on health, there are many studies reporting contradictory opinion about (Wuttke *et al.*, 2002). The first consideration is that isoflavonoids needs to be converted by gut bacteria from their aglycones form in equol in order to explain their beneficial effects (Setchell *et al.*, 1984), but, not all the subjects are in position to produce free aglycones from isoflavones glycoside due to their gut flora

activity. Moreover, because of the same reason, they are not able to convert isoflavones in equol (Zubik and Meydani, 2003).

A further topic that must be considerate is the influence of placebo effect on the results reported by many studies on the healthy properties of isoflavonoids. Therefore, some researchers performed new experiments using a double blind placebo-controlled study design. Barber *et al* reported no significant differences in reduction of hot flushes in menopausal women fed either with isoflavones or placebo (Baber *et al.*, 1999).

Regarding the effect of isoflavonoids on cancer, the administration of isoflavonoids in menopausal age is not enough to obtain the beneficial effect. Maskarinec *et al.* reported that breast cancer occurrence is lower only in women that used to eat food rich in isoflavones during their childhood. Thus, women who started the intake of isoflavones later in their life do not take advantage against breast cancer development (Maskarinec and Meng, 2001). Therefore, data on the beneficial or adverse effect of isoflavones on mammary cancer remain contradictory.

Isoflavonoids may also have a toxic effect according to Seidlova *et al*. study. Estrogenic supplements cause desquamation and cornification of the vaginal epithelium, resulting in the typical oestrous vaginal smear. Similar effect occurs in the vaginae of postmenopausal woman with phytoestrogens as isoflavonoids dietary intake (Rimoldi *et al.*, 2007).

In addition, despite phytoestrogens intake decreases the lipid content in blood, there still is an increased incidence of both fatal and non-fatal cardiovascular events in subjects under estrogens and phytoestrogens administration (Wuttke *et al.*, 2002). Anyway, a combination of isoflavones and protein is necessary to have a significant effect on lipids and arterial parameters (Wuttke *et al.*, 2002).

In conclusion, despite negative effects of isoflavonoids are not so relevant, further studies are required to confirm their real beneficial effects.

3.5.2 Bioavailability of flavonoids and isoflavonoids

All the beneficial effects of isoflavonoids depend on the concentration of these compounds that will be absorbed by the organism (Vitale *et al* ., 2013).

Bioavailability is definite in pharmacological sense as the proportion of compound administered orally, which appears in plasma over time. In other words, it represents the proportion of compound that is absorbed from the gastrointestinal tract (Birt *et al.*, 2001).

The bioavailability of the flavonoids and isoflavonoids showed to be influenced by their chemical form in food, their hydrophobicity, susceptibility to degradation, microbial flora of the consumer and food matrix (Birt *et al.*, 2001).

Hydrophobicity is one of the most important factors that influence the bioavailability of many compounds. Generally, lipid-soluble compounds are not directly excreted in urine by the organism, but they appear as their more water-soluble metabolites. Furthermore some fraction of lipid-soluble compounds would be stocked in fat store. Thus, ingestion versus excretion will not tell the full story of the biological fate of those compounds (Hendrich, 1994).

Flavonoids and isoflavonoids are not very soluble neither in water nor organic solvents; they are not completely soluble even in their most appropriate solvents (ethanol, methanol, acetonitrile) (Coward *et al.*, 1993). Flavonoids and isoflavonoids are usually present as glycoside forms, which undergo enzymatic cleavage of sugar moiety by mammalian or microbial glycosidase. Family of UDP-glucuronosyl transferases acts on the phenolic moieties of isoflavonoids to produce the glucuronidides in liver (Watanabe *et al.*, 1998). These metabolites are more readily transported in the blood and excreted in bile or urine than their parents aglycones (Wu *et al.*, 2011). Indeed, isoflavones from fermented soy foods, such as tempeh, natto or miso would be somewhat more

rapidly absorbed because the isoflavone aglycones content would be relatively increased in comparison with unfermented soy food (Xu *et al.*, 2000).

The bioavailability of food and its compounds does not depend only on the hydrophilicity of the compound but also on the possibility to be degraded. For example, genistein seems to be more retained than daidzein. But actually it undergoes gut microbial degradation thus it is not found in urine (Hendrich, 1994; Zhang *et al.*, 1999)

The absorption of isoflavonoids is also influenced by the molecular weight (Kano *et al.*, 2006). Isoflavone aglycones have appropriate molecular weight for gut absorption, around 250 g/mol, which allows the diffusion in the gastrointestinal tract. Isoflavones glycosides are dominant in food, but they are found in the urine differentially than algycones. It means that glycosides are somehow transformed into aglycones. Human intestine or gut microflora glycosidases seemingly cleave these moieties before the isoflavones will be absorbed (King and Bursill, 1998).

Some isoflavonoids can be transformed into equol by the microbial biotransformation (Setchell *et al.*, 1984). Isoflavones are also transformed in other molecules like O-desmethylangolesin (ODMA) during gut fermentation. The gut microbial diversity seems to be at least partially responsible for major difference in isoflavone metabolism among individuals (Kelly *et al.*, 1993). Anyway, isoflavones and flavonoids-degrading microorganism have been partially identified but the knowledge is still patchy. Furthermore, the bioavailability of dietary constituents depends to some extent upon interaction with other dietary components.

Studies showed that the administration of 40 g of dietary wheat fibre during a single day significantly suppressed urinary geinstein excretion by 20% after a soy milk meal compared with 15 g dietary fibre (Tew *et al.*, 1996). It must be understood that a high carbohydrate milieu causes increased intestinal fermentation, which would enhance phytoestrogens converting strains abundance increasing the formation of equol (Setchell and Cassidy, 1999)

Thus, wheat fibre has only a modest effect on isoflavones bioavailability and the effects of other dietary factors on isoflavones bioavailability remain to be studied.

3.5.3 *Major flavonoids in Leguminosae*

Leguminosae are the plant family characterized by high occurance of isoflavonoids (Farag *et al.*, 2007). Among the Leguminosae species, *Glycine max* L. is probably the mostly associated to the high content of isoflavoids. Specifically, soy is particularly rich daidzein and genistein which belong to the class isoflavonoids, a sub-class of flavonoids (Klejdus *et al.*, 2004). The high concentration of these isoflavones is probably due to the higher expression of the gene encoding for the IFS enzyme which lead to their biosynthesis (Dixon and Ferreira, 2002).

Other legume species famous for its flavonoids content is *Trifolium pratense* L.. As well as *G. max* L.*, T. pratense* L. is rich in isoflavones but it is differentilly characterized by an high content of biochanin A and formononetin (Klejdus *et al.*, 2001).

This study focuses on the analysis of isoflavones in *Medicago sativa* L. This plant is also rich in flavones such as apigenin, which are present as precursors of the biosynthetic pathway. Alfalfa contains isoflavonoids such as medicarpin, a pterocarpan, and coumestrol, which belong to the group of coumestans. Pterocarpan and coumestan are differentially synthesized and accumulated after pathogen attack and leaf wounding, respectively (Dixon and Paiva, 1995; Farag *et al.*, 2007).

The chemical structures of major isoflavonoids are reported in Fig.4.

Glycine max L.
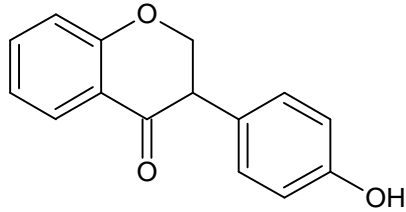
Daidzein	Genistein
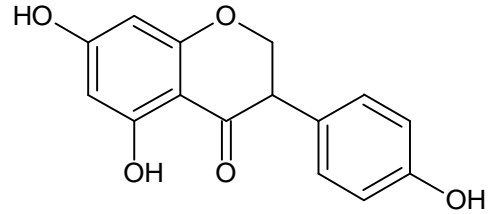
Trifolium pratense L.

Biochanin A	Formononetin
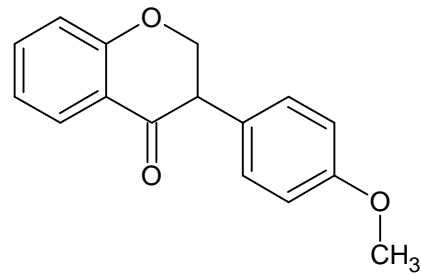
Medicago sativa L.

Apigenin	Medicarpin
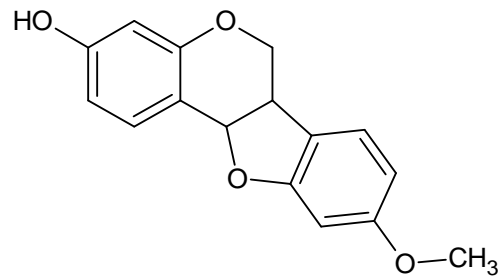
Coumestrol
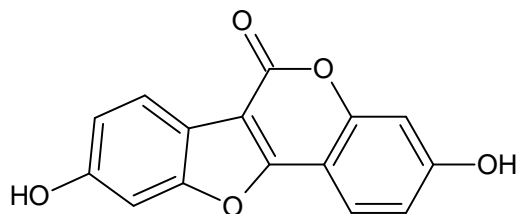
Figure 4 Molecular structures of the major flavonoids and isoflavonoids in *Glycine max* L.,

***Trifolium pratense* L. and *Medicago sativa* L.**

3.6 *Chemical analysis of isoflavonoids and flavonoids*

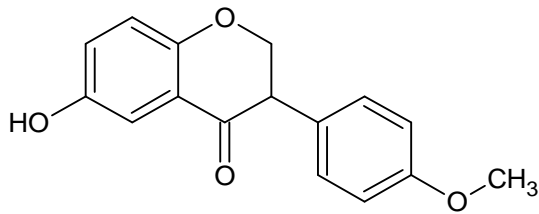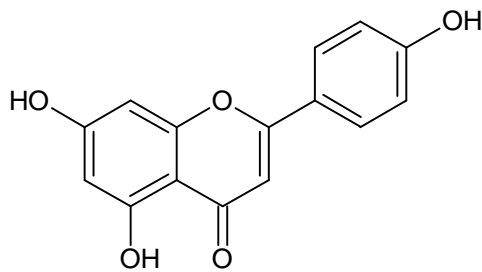Since their beneficial effect on human health is known, analytical procedures have been developed in order to identify and measure the isoflavonoids present in food and plant material. These procedures include extraction, sample treatment, separation and detection.

Extraction

Extraction is a fundamental step for recovery and isolation of phytochemicals from raw material. It is influenced by several factors such as chemical nature of the compounds of interest, their interaction with the matrix and the employed extraction method. Among the methods used for the flavonoids extraction, Soxhlet extraction is the most frequently worldwide used for solid samples by using aqueous methanol or acetonitrile solution as solvent. This method is also influenced by pH of the solvent, temperature, time and sample-to-solvent volume ratio (Stalikas, 2007). Despite its common use, Soxhlet extraction has lower extraction efficiency if compared with other methods such as ultrasound-assisted- or microwave-assisted-extraction which can be also used for the same purpose (Gao and Liu, 2005).

Supercritical fluid extraction is also used as extraction methods for isoflavonoids. This technique is the more specific if compared to Soxhlet- or ultrasonic extraction because it avoids the extraction of chlorophyll and other no polar compounds in the extract (Stalikas, 2007), although it is the less efficient (Rostagno *et al.*, 2002).

Sample treatment

In order to obtain an accurate identification and quantification of the flavonoids and relative derivate, a proper sample treatment is necessary. Hydrolysis is a pre-treatment of the extract that can simplify the identification

of isoflavonoids conjugates. It may be carried out both in acid or in basic conditions although an enzymatic procedure is also suitable. The basic hydrolysis acts on ester bond removing the malonyl/acetyl group that are linked to the sugar moiety. The acid hydrolysis is usually performed using inorganic acid such as HCl under high temperature (Delmonte *et al.*, 2006). Both acid conditions and temperature act on the glycosidic bond removing the sugar moiety and producing free aglycones. The comparison of the chromatograms relative to the raw extract and the hydrolyzed samples leads to the identification of the esters and the glycosides in basic- and acid conditions, respectively (Delmonte *et al.*, 2006; Rostagno *et al.*, 2009).
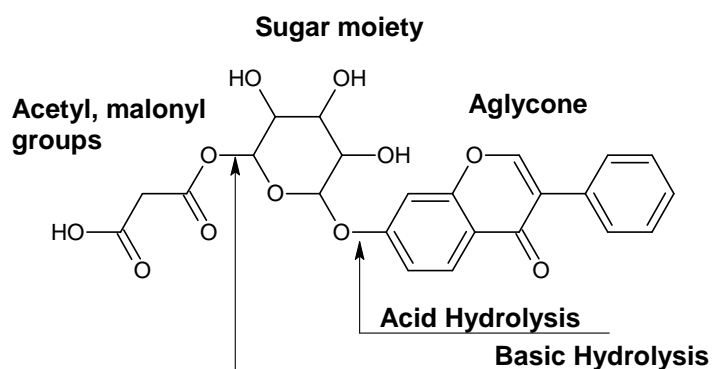
Figure 5 Scheme of acid and basic hydrolysis of isoflavonoids derivates.

Separation

Liquid chromatography coupled with mass spectrometry detector or diode array detection is far the most used methods in the detection of isoflavonoids in crops. Besides, various methods can be employed among them, an immunoassay is worth mentioning (LapčíK *et al.*, 1999), gas chromatography, capillary electrophoretic and capillary electrochromatographic methods are also used but not so commonly (Stalikas, 2007).

In chromatographic systems, flavonoids are retained by the stationary phase according with the interaction of their substituent and the stationary phase (Klejdus *et al.*, 2008). Since most flavonoids have similar chemical properties they can be eluted almost in the same time. However, the peaks must be well

separated in order to succeed in the quantification. The grade of separation of the peaks is called resolution factor and is identified by Rs. A good optimization of the method is needed in order to obtain well separated peaks.

Detection

All flavonoids contain at least one aromatic ring, thus they absorb UV light. Usually the first maximum in the UV spectra is due to the A-ring is found in the 240-285 nm range. The second maximum is attributed to the substitution pattern and conjugation of the C-ring and appear in the 300-350nm range (Stalikas, 2007).

Diode array detector (DAD) gives a complete UV or UV visible spectra to all the sample components when they elute from the column. Results consists of a three dimensional graphic in which one axis is relative to the absorption, one to the retention time and the last to the wavelengths. The obtained spectra from the chromatogram can be matched with a library of standards for identification purposes. A co elution of compounds results in not clear spectra (Mattila *et al.*, 2000).

Mass spectrometer (MS) is a method based on the study of the ionized particles generated after the ionization, submitted to magnetic field (Rouessac and Rouessac, 2007). It can be used for the detection of isoflavonoids aglycones, their derivates or glycosides, and their other conjugates. Each individual isoflavone is characterized by its molecular ion generated after the ionization, and by its specific fragmentation products (Klejdus *et al* ., 2004; Vacek *et al* ., 2008). This technique can be applied to the isoflavonoids analysis in order to obtain an accurate identification and structure elucidation (Stalikas, 2007).

Optimization of the method

All the chromatographic analysis aims to obtain adequate peaks separation in the chromatogram in the shortest time possible. With this purpose, the

chromatographer should find out the right conditions for his analysis. Computer-controlled HPLC system helps in managing all the parameters of the analysis including temperature, flow rate and mobile phase gradient program. Generally is useful first to do a very slow chronographic run with a low flow rate and a linear gradient of two components mobile phase in order to figure out the ratio of mobile phase in which the compounds of interest are eluted (Snyder *et al.*, 2011). Then an isocratic part in the mobile phase gradient program may be inserted in order to improve the separation quality. This is the way followed in this work.

4 Material and Methods

4.1 Overall experimental approach

The aim of the work was to determine the effect of alfalfa cultivar on the concentration of a major isoflavone coumestrol, flavonoid apigenin and other accompanying flavonoids. Samples of alfalfa from 15 varieties from three plots were collected and dried.

Dried samples were extracted in a Soxhlet-type apparatus and evaporated. HPLC method was developed to quantify the content of apigenin and coumestrol aglycones. Moreover, relative peak areas of major peaks of the chromatogram were exported for PCA and RDA statistical analysis. The tentative nature of these peaks was tentatively identified based on their behaviour under the acid- and basic-hydrolysis conditions and by interpretation of their MS-spectra. Moreover, both apigenin and coumestrol identity was verified on LC-MS and this method was also used for a detailed quantification of a set of approx. 40 phenolic acids, flavonoids and isoflavonoids in one selected alfalfa sample.

A graphical overview of the analytical approach might be seen in Fig. 6.
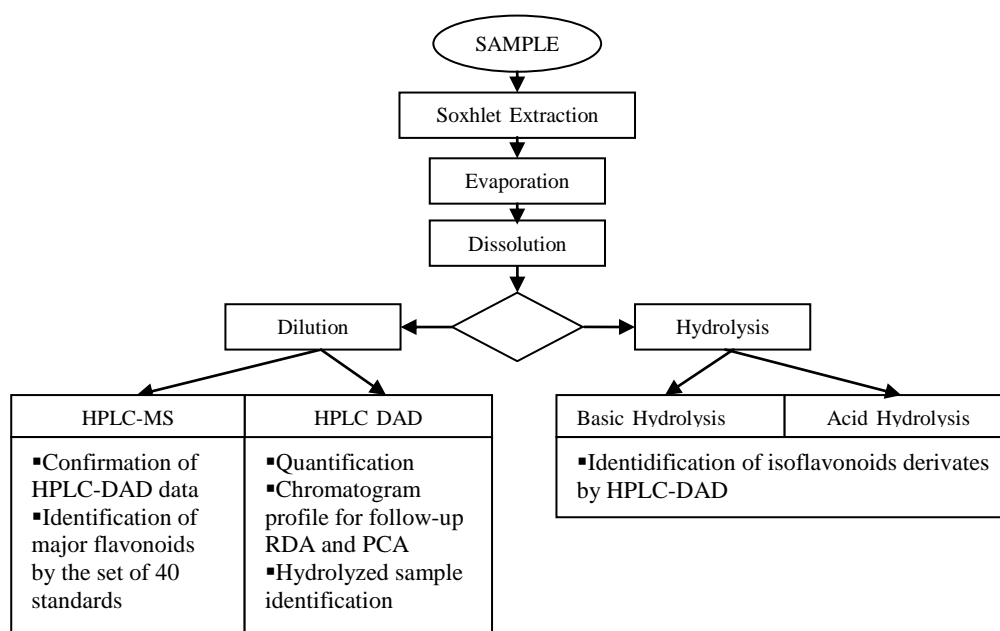
Figure 6 Flow chart of the experimental approach followed in for the thesis purpose

4.2 Plant material

Samples of aerial parts of 15 alfalfa cultivars, each consisting of approximately 50 g (DM) were collected, each in three biological replicates. They included Czech cultivars *Palava*, *Morava*, *Vlasta*, *Magda*, *Jarka*, *Zuzana*, *Jitka*, *Niva*, *Oslava*, *Kamila, Litava, Kamila, Tereza*, *ZE XLII* and the French cultivar *Europe*. The material originated from the Research station of the Czech University of Agriculture in Cerveny Ujezd and was obtained as part of a larger experiment on alfalfa management practices done by Dr Josef Hakl. Further information on these cultivars is available in (Hakl *et al.*, 2012). The varieties differed in various parameters, among them, only three were known as nitrogen fixators (Niva, Oslava and Zuzana).

The mean annual temperature at the location is 7.7 °C and the long-term annual sum of precipitation is 493 mm. The prevailing soil type is clay loam orthic luvisol and the kind of soil is medium with the neutral soil reaction. The plants were grown without any herbicides used. Investigated cut was the

second cut in 2009 (1.7.2009) of four cut management. The plants were in late bud stage.

Palava provided the lowest yield (considered as dry matter) in comparison with new lucerne varieties but this was not significantly different ($P<0.05$) (data not shown).

All samples were evaluated separately on their proportion of stem sizes, densities, yield and dry mater and these data are present in Table 1.

Table 1 Characteristics of the evaluated plants.

Sample N°	Variety	Stems/m²		stem density (stems/m²)	maximal stem length (cm)	average weight of 1 stem	yield of sample g/m2	DM
		> 20 cm lenght	< 20 cm lenght					
A16II9	Palava	1125	75	1200	89	1.217	1460	0.319
A17II9	Morava	825	125	950	97	1.016	965	0.295
A18II9	Vlasta	650	75	725	89	1.090	790	0.277
A19II9	Magda	650	0	650	99	1.346	875	0.249
A20II9	Jarka	825	125	950	102	1.211	1150	0.466
A21II9	Zuzana	700	25	725	88	1.290	935	0.318
A22II9	Jitka	725	25	750	105	1.823	1368	0.270
A23II9	Niva	825	0	825	102	1.333	1100	0.292
A24II9	Oslava	750	100	850	90	0.974	828	0.313
A25II9	Europe	600	75	675	98	2.122	1433	0.305
A26II9	ZE XLII	600	0	600	91	1.933	1160	0.349
A27II9	Litava	675	125	800	100	1.316	1053	0.298
A28II9	Kamila	675	50	725	102	1.679	1218	0.280
A29II9	Tereza	475	50	525	96	1.510	792.5	0.505
A30II9	Holyna	625	50	675	81	0.981	662.5	0.255
B16II9	Europe	900	150	1050	89	0.819	860	0.351
B17II9	Jarka	650	100	750	90	1.023	767.5	0.205
B18II9	Jitka	700	125	825	91	0.676	557.5	0.105
B19II9	Niva	600	75	675	87	0.926	625	0.195
B20II9	Kamila	825	100	925	85	0.949	877.5	0.357
B21II9	Vlasta	875	100	975	93	0.921	897.5	0.199
B22II9	ZE XLII	825	75	900	93	1.014	912.5	0.332
B23II9	Tereza	950	25	975	88	0.833	812.5	0.095
B24II9	Morava	725	75	800	92	0.828	662.5	0.350
B25II9	Zuzana	1075	100	1175	88	0.800	940	0.261
B26II9	Holyna	950	125	1075	85	1.237	1330	0.261
B27II9	Oslava	650	0	650	87	0.962	625	0.195
B28II9	Palava	800	75	875	91	0.966	845	0.305
B29II9	Magda	925	100	1025	96	1.005	1030	0.235

Sample N°	Variety	Stems/m²		stem density (stems/m²)	maximal stem length (cm)	average weight of 1 stem	yield of sample g/m2	DM
		> 20 cm lenght	< 20 cm lenght					
B30II9	Litava	775	25	800	86	1.075	860	0.268
C16II9	Oslava	825	125	950	79	0.705	670	0.214
C17II9	Kamila	825	125	950	93	0.979	930	0.198
C18II9	ZE XLII	825	125	950	73	0.558	530	0.281
C19II9	Tereza	675	150	825	78	0.873	720	0.372
C20II9	Palava	900	300	1200	83	0.858	1030	0.247
C21II9	Litava	950	175	1125	83	0.647	727.5	0.240
C22II9	Europe	725	50	775	90	0.881	682.5	0.214
C23II9	Holyna	550	50	600	90	1.058	635	0.302
C24II9	Magda	550	200	750	87	0.727	545	0.298
C25II9	Jitka	700	50	750	89	0.877	657.5	0.320
C26II9	Jarka	1100	75	1175	97	0.960	1127.5	0.209
C27II9	Vlasta	750	125	875	89	1.060	927.5	0.269
C28II9	Zuzana	800	175	975	78	0.905	882.5	0.312
C29II9	Niva	875	100	975	90	0.851	830	0.237
C30II9	Morava	625	125	750	90	1.323	992.5	0.302

DM, Dry Matter

4.3 Chemicals

Standards compound for the HPLC-DAD analysis (flavon, coumestrol and apigenin) were purchased in best available purites from SIGMA-ALDRICH (CZ). The reagents for the hydrolysis such as NAOH, HCl (37%) were from Lach-Ner (CZ) Company, as well as methanol (99.9%, MeOH) for the extraction. Orthophosphoric acid (85%), acetic acid (99.7%), triethylamin (TEA, 99%), trifluoracetic acid (TFA, 99%) and HPLC gradient grade acetonitril CHROMASOLV$^®$ were from SIGMA-ALDRICH (CZ). Water used was Millipore Milli-Q deionised ultrafiltered water (Merck, DE).

4.4 Extraction

The Soxhlet technique of extraction is the most common method for the extraction of the isoflavonoids from soybean samples (Rostagno *et al* ., 2009) and is suitable for alfalfa. Parameters as solvent polarity, temperature and duration of the may affect the extraction process.

The method was previously described by (Leuner *et al.*, 2013). The extraction was carried out using the 80% MeOH in a Soxhlet-like extractor MEZOS SER148 (VELP Scientifica, IT) at the temperature of 230°C for 80 min. An amount of 2.5g of each sample was introduced into a cellulose cartridge and enclosed with cotton for the extraction.

The cartridge was immersed in the solvent for 60 min and then refluxed with the solvent The obtained solution was evaporated *in vacuo* at 40°C using the rotary evaporator (Heidolf, DE). The dry residue of the extract was weighed, dissolved in 10 ml of the 80% aqeuous methanol for analysis or hydrolysis procedure, and stored at room temperature until analysed.

Before analysis, samples were filtered through a 22 μm PTFE filter, diluted 1:5 in 80% aqueous methanol solution and injected in the HPLC.

4.5 HPLC-DAD analysis

HPLC separation was performed on Kinetex Phenyl phase PFP column (2.6μm, 100A, 150x3.00 mm) from Phenomenex (US). The system consisted of HPLC Dionex P680 pump, thermostatic column compartment TCC-100 and UVD340U detector and Waters 717 autosampler.

The optimum separation was achieved under gradient conditions within 90 minutes (for gradient, see Tab. 2), which was followed by the equilibration to original conditions. Sample amount injected was 40 μL, flow rate 0.6 mL/min, column temperature 30°C. The integration of the peaks was carried out in Chromeleon v. 6.8 software at the wavelength of 250 nm for all analytes except for flavon which was detected at 300 nm. For compounds where the standards were not available, peak areas of unidentified peaks were used for a follow-up statistical tests and the nature of these compounds was tentatively identified based on their behaviour under basic and acid hydrolysis and based on MS spectrum interpretation (see below).

Statistical analyses of the data obtained from the quantification were done. In order to detect the significance of difference (P<0.01 or P<0.5) of variables a one way ANOVA test was carried out.

4.6 HPLC-DAD Method optimization

The chromatographic conditions needed to be optimized to achieve good separation between two critical peaks of apigenin and coumestrol. Using the previously published method in (Leuner *et al.*, 2013), both compounds co-eluted. Thus, three methods were developed using the Gemini C18 column, achieving the Rs ranging from 0.4 to 2.8. However, none of these methods proved to be achieve optimum resolution of both critical peaks. Later, usage of Kinex PFP column under conditions stated in Tab. 2 was shown as optimal.

Table 2 HPLC conditions and different mobile phases' gradients.

Column	Solution A	Solution B	Gradient (% of A, B= 100-A)	Flow rate (ml/min)	T (°C)	Rs[*]	Fig.
Gemini C18	ACN	0.1%TFA	5% → 30%(13min) → 32% (50min) → 100% (80min)	0,8	30	0.4	A
Gemini C18	ACN	0.04% H3PO4 + 0.09% TEA, pH6	5% → 50% (60min)	0,8	30	1.1	B
Gemini C18	ACN	0.04% H3PO4 + 0.09% TEA, pH6	5% → 26% (40min) → 31% (70min) → 50% (90min)	0,8	30	2.8	C
Kinetex PFP	ACN	Acetic acid 0.1%	5% → 28% (35min) → 32% (60min) → 100% (80min)	0.6	30	4.9	D

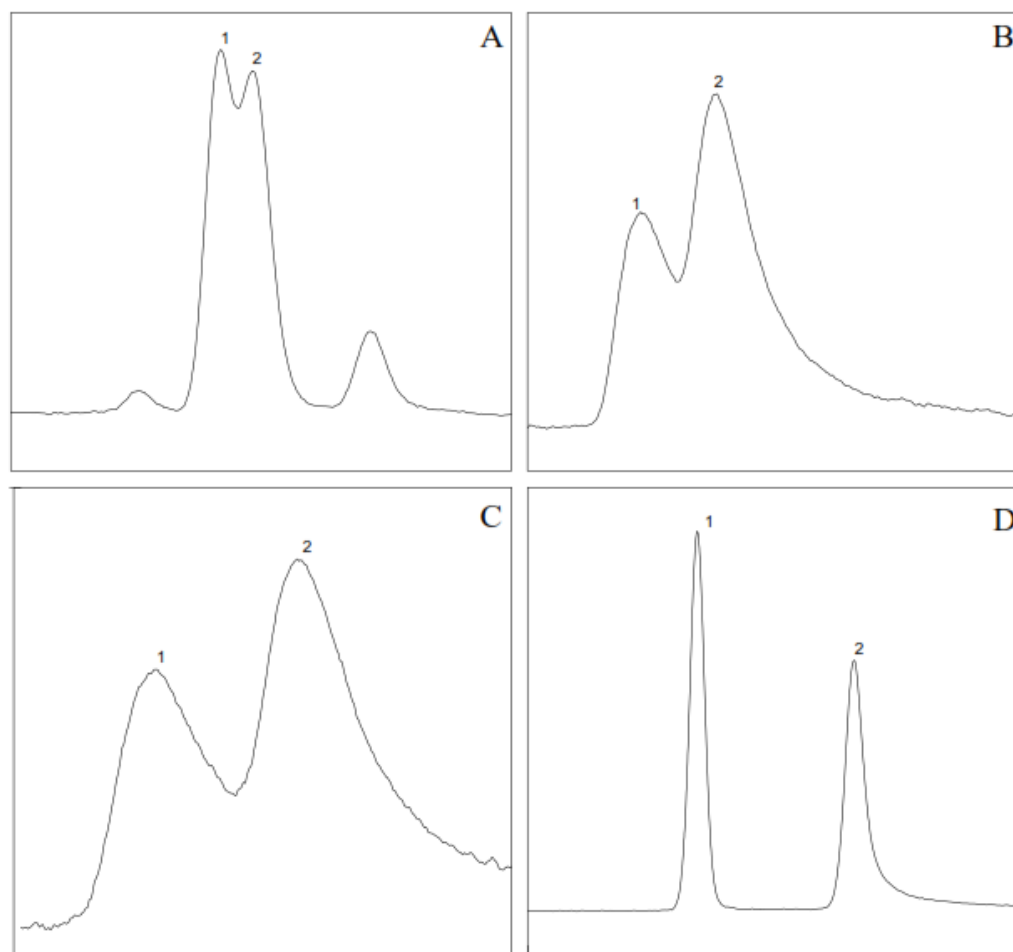[*]Rs, Resolution of the two critical peaks coumestrol and apigenin

Figure 7 Resolution of the critical peaks of interest coumestrol (1) and apigenin (2) within the tested mobile phase program reported in Table 2 identified as A, B, C, D.

4.7 Identification of conjugates

To further understand the exact conjugation (presence of either sugar moieties or ester-bound malonyl or acetyl groups) of the compounds of interest, the crude extract was hydrolysed by acid and basic hydrolysis.

The acid hydrolysis was performed using approximately 4.5 mL of the soxhlet extrac. The extract was put in a closed flask with 2.7 mL of HCl 12N and left in the dryer at 110°C for 1h (Delmonte *et al.*, 2006).

The solution was cooled at room temperature at the later stage and then neutralized a pH 2.5 by NaOH 2N. The obtained solution was stoked in a closed plastic tube.

The basic hydrolysis was performed using 4.5 mL of the same extract mixed with 50 µL of a solution of NaOH 2N. Furthermore ,100 µL of glacial acetic acid were added after 10 min (Delmonte *et al.*, 2006).

After hydrolysis, samples were diluted 1:5 with 80% methanol solution and filtered with a through a 0.22 µm PTFE filter before the HPLC analysis. After analysis, chromatograms were compared on changes in peak profiles. While acid hydrolysis leads to a complete deconjugation and leaves out only the aglycon, the basic hydrolysis leads to break of the ester bonds of the malonyl or acetyl groups from the sugar moiety and leaves out the glycosides. Comparison of the chromatograms enabled us to tentatively identify the structures.

4.8 LC-MS verification and quantification of phenolics in the extracts

To confirm the identity and purity of the peaks in our DAD chromatograms, LC-MS analysis was carried out. An UHPLC Agilent series 1290 Infinity instrument coupled to Agilent triple quadrupole mass spectrometer 6460 with a Jet Stream ESI ion source was used. The method was previously developed and performed by Petra Miksatkova (Miksatkova *et al* ., in press). Two analytical approaches were selected. Firstly, same method as that used by us on HPLC, using the same Phenomenex (Torrance, CA, USA) Kinex PFP column (2.6µm, 100A, 150x3.00mm) was used on the LC-MS system, and second, analytical method recently developed by Dr Miksatkova, optimized for the detection and quantitation of 54 known phenolic compounds. During the first mentioned analytical approach, MS detection has been done in positive mode for m/z 100-500 and negative mode for m/z 100-500. During the

second, ionization modes were optimized for every time point and the gradient and also column differed. The quantification was done according to calibration curves obtained from external previously purchased pure analytical standards.

Ion Source parameters were the same for both methods: drying gas temperature: 300°C; drying gas flow: 4 l/min; sheath gas temperature: 380°C; sheath gas flow: 10 l/min; nebulizer pressure: 35 psi; capillary voltage: 3500V; nozzle voltage: 2000 (positive mode) and 500 (negative mode); fragmentor: 100 V.

4.9 Method validation

Recovery determination was based on comparison of theoretical concentrations in the sample obtained by spiking a blank sample with 20µg of flavon, apigenin and coumestrol standards with their real concentrations obtained from the HPLC-DAD analysis. Reproducibility was performed by comparing peak quantities in three independent inter-day extractions. Limit of detection was determined as the least concentration that led to a detector response at the wavelength of 250 and 300 nm, respectively which was three-times larger than the noises. Limit of quantification was determined as five-times multiples of the noise.

5 Results and discussion

5.1 LC-MS quantitative analysis of sample B25II9

An LC-MS analysis was performed in one selected sample of our sample set in order to confirm HPLC-DAD results with an independent method. Moreover, LC-MS method was used to confirm identity of minor peaks from our HPLC-DAD chromatogram.

Table 3 Quantification of 54 selected phenolics of sample B25II9 variety Zuzana in LC-MS

compound	µg/g	compound	µg/g
anisic acid	8.69	prunetin	-
caffeic acid	4.81	puerarin	-
chlorogenic acid	-	sissotrin	T
p-coumaric acid	**132.0**	sophoricoside	12.71
ferulic acid	**57,9**	tectoridin	T
gallic acid	2.07	tectorigenin	-
salicylic acid	**759.3**	5.7.3´.4´-tetramethoxyisoflavon	-
sinapic acid	2.33	6.7.4´-trihydroxyisoflavone	-
syringic acid	18.89	5.7.4´-trimethoxyisoflavone	T
vanilic acid	**65.18**	7.3´.4´-trimethoxyisoflavone	0.08
epicatechin	-	apigenin	**65.42**
4-hydroxycoumarin	-	apigenin-7-*O*-glucoside	9.32
pterostilbene	-	hesperetin	-
resveratrol	T	7-hydroxyflavone	-
scopoletin	T	isoquercitrin	2.51
biochanin A	0.54	kaempferol	11.29
coumestrol	**68.2**	liquiritigenin	9.21
daidzein	T	luteolin	12.88
daidzin	0.14	luteolin-7-*O*-glucoside	T
7.4´-dimethoxyisoflavone	9.54	morin	-
equol	-	myricetin	-
formononetin	**30.8**	naringenin	0.08
genistein	T	naringenin-7-*O*-glucoside	5.87

To be continued

compound	µg/g	compound	µg/g
genistin	-	naringin	-
glycitein	5.73	quercetin	2.49
glycitin	**5.99**	quercetin-3-*O*-arabinoside	-
7-hydroxyisoflavone	-	rutin	1.79

T, traces (<LOQ[*]), -, (<LOD), *LOQ present in Tab. 3.Concentration is expressed per gram of dry matter.

Our LC-MS analysis, which was set up for the detection and quantification of 54 phenolic compounds, revealed the presence of 37 compounds. Out of them 29 could be quantified. Phenolic acid represented the largest chemical group in our extract. The most abundant compound was salicylic acid followed by *p*-coumaric acid and ferulic acid with the concentration of 759.3 µg/g, 132.0 µg/g and 57.9 µg/g, respectively. *p*–coumarate is a precursor of flavonoids and isoflavonoids synthesis. Moreover, glycosides and aglycones of flavonoids and isoflavonoids were detected. Out of the glycosides, apigenin-7-O-glucoside was the most abundant, followed by the glycitin which is present in concentration of 9.32 µg/g and 5.99 µg/g, respectively. For aglycones, coumestrol and apigenin turned out to the relative higher concentration in our extract. Thus, these compounds were further subject to the quantification on the HPLC-DAD system. Besides formononetin, biochanin A, glycitein, were detected in the concentration of 30.8 µg/g, 0.54 µg/g, 5.73 µg/g respectively. Many other phenolic compounds were detected but their concentrations have not been considered.

## 5.2 *Quantification of major constituents in alfalfa extracts*

Apigenin and coumestrol were available as pure standards and were thus determined quantitatively in all samples. The method developed within this study showed LOD 10 ng/g for apigenin and 20 ng/g of dry matter for coumestrol. The LOQ was 17 ng/g and 35 ng/g for apigenin and coumestrol,

respectively. The recovery of the compounds ranged from 107% to 110% for coumestrol and apigenin respectively. The inter-day variation of the method including three independent measurements and determined as standard deviation was 11.9%.

Table 4 Limit of detection (LOD) and of quantification (LOQ) of selected compounds

Compound	LOD (ng/g)	LOQ (ng/g)	Wavelength (nm)
Flavon	10	17	350
Coumestrol	20	35	300
Apigenin	40	65	300

Coumestrol and apigenin were quantified in the samples after external calibration with coumestrol and apigenin standards. Calibration curves are shown in Fig. 8.
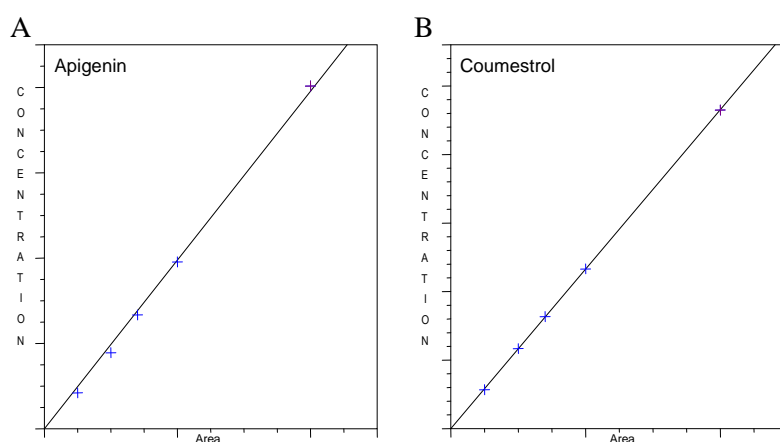
Figure 8 Calibration curves of apigenin (A) and coumestrol (B). Concentration used: 2,5-5-7,5-10-20 µg/mL

Results from the HPLC-DAD quantification were recalculated considering all dilutions steps during sample preparation and original dry matter of the plant material. The content of apigenin ranged from 42.8 µg/g of ZE XLII to 94.0 µg/g of Holyna, Morava and Zuzana. While, the content of coumestrol ranged from 15.5 µg/g of Jarka to 52.2 µg/g of Morava. Data shown in table 5.

Table 5 Contents of coumestrol and apigenin in the selected varieties.

	Apigenin		Coumestrol	
	Mean (µg/g)	Standard Deviation	Mean (µg/g)	Standard Deviation
Europe	63.6	23.5	39.5	4.7
Holyna	94.0	27.8	40.4	6.7
Jarka	50.9	33.0	15.5	9.3
Jitka	56.4	33.4	25.8	10.7
Kamila	74.4	50.5	24.4	7.8
Litava	72.2	11.8	45.2	27.4
Magda	80.1	20.3	34.4	23.7
Morava	94.0	20.2	52.2	15.1
Niva	68.7	44.1	37.6	25.2
Oslava	72.0	14.7	39.1	10.1
Palava	91.9	9.5	36.8	13.3
Tereza	89.9	29.5	27.5	13.1
Vlasta	93.2	20.6	23.0	2.3
ZE XLII	42.8	13.7	31.9	8.4
Zuzana	94.0	8.0	44.5	20.6

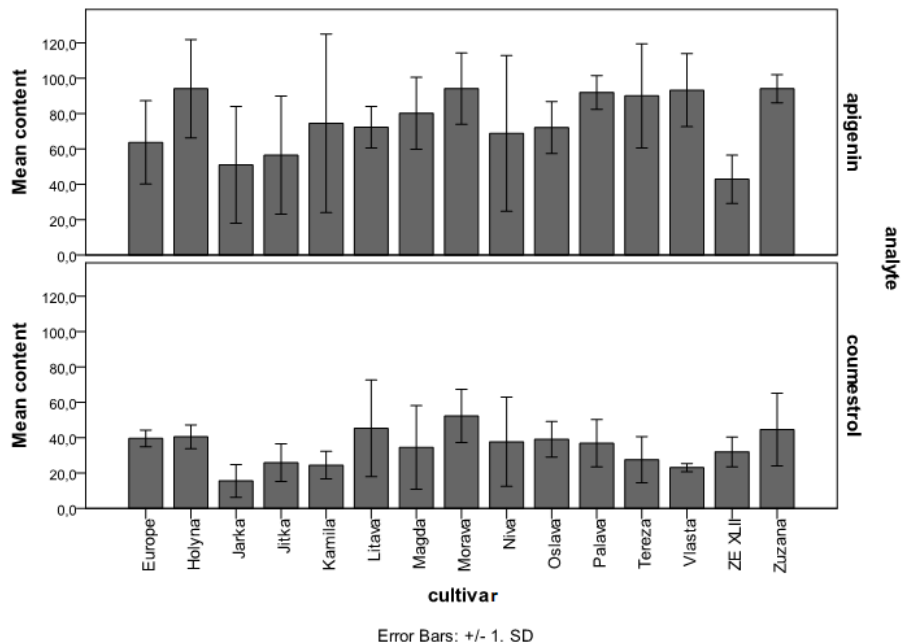A graphic representation of the obtained data is reported in fig.9.

Figure 9 Histogram of apigenin and coumestrol contents in evaluated alfalfa varieties.

5.3 HPLC-DAD profiles and RDA/PCA Analysis

The mobile phase gradient method performed led to an elution order of the phenolic compounds which followed a sequence of decreasing polarity, where the flavonoids diglucosides eluted first, followed by monoglucoside, acylated monoglucosidesd, and free aglycones as is shown in Fig.10.
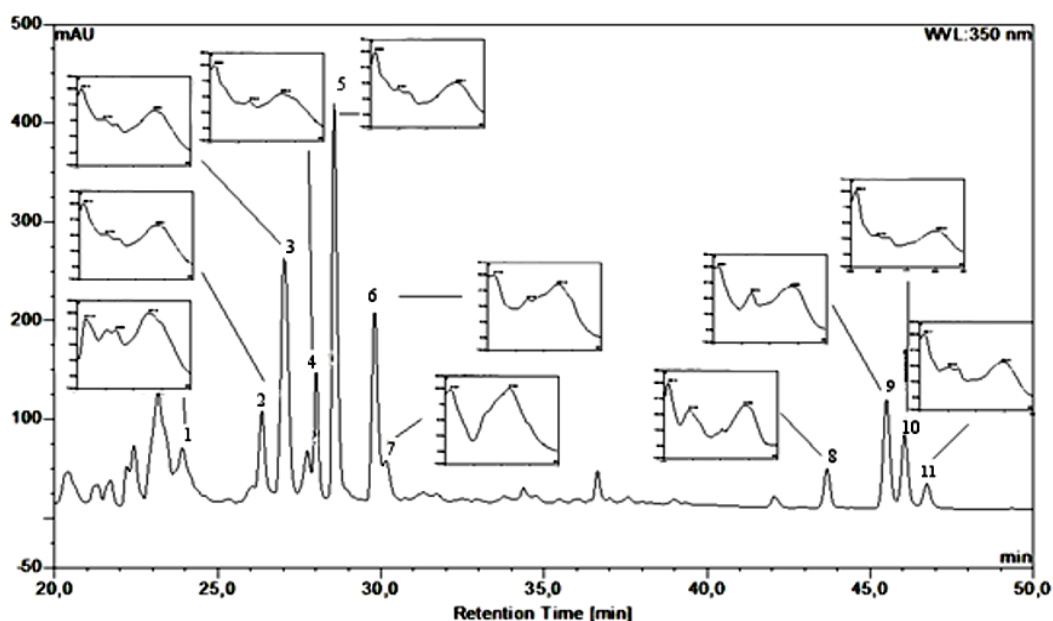

Figure 10 Chromatogram of sample C19II9 with the spectra of peak compounds.

Table 6 Peaks list of the chromatogram in Figure 10.

Peak's n°	Compound	Retention time (min)	[M+H]+*
1	Unknown Glycoside 1	23.9	(319-433)
2	Unknown Glycoside 6	26.3	(373-472)
3	Unknown Glycoside 2	26.9	(279-436)
4	Unknown Glycoside	27.7	(350-392.436)
5	Unknown Glycoside 3	28.0	(271-477)
6	Unknown Glycoside 4	29.9	(306-342.447)
7	Unknown Glycoside 5	30.2	(257-317-466)
8	Coumestrol	42.6	269
9	Apigenin	44.3	271
10	Unknown Compound	44.8	(283-301)
11	Formononetin	45.5	269

*= Molecular weight plus proton as identified by LC-MS.

From the chromatogram eleven peaks has been identified. These peaks were representatives of all the samples and they are easily identifiable.

The chromatogram can be divided in two parts, the left side which contains the first major peaks and the right part which contains the last four peaks in the picture. The peaks with the smaller retention time are relative to flavonoids derivates such as glycosides and esters.

Since this work is focused on apigenin and coumestrol determination, an HPLC run was performed injecting the standards of apigenin and coumestrol in order to determine their retention time and thus identify their correspondent peaks in the extract's chromatogram (data no shown).

Most peaks of the HPLC-DAD chromatogram could not be identified by retention time or by UV spectra or because of the unavailability of all the standards. Thus, tentative identification by determining their properties was done on the basis of their behaviour after acid- and basic hydrolysis of the crude extract. Here we assumed the all acetylated and malonylated conjugates of flavonoids glycosides disappeared from the chromatogram and may appear as their corresponding glycosides after the basic hydrolysis treatment.

Similarly, both glycosides and acetylated/malonylated conjugates disappeared after acid hydrolysis and appear as their corresponding aglycones. This way all major peaks in the representative chromatogram of the sample C19II9 were tentatively identified either as glycosides or aglycones.

During the acid hydrolysis, protons go to the oxygen atom of the glycoside bound triggering the break of that and making the aglycone free of the glycoside moiety of the initial molecule. While the basic condition lead to the break of the ester bound (Delmonte *et al.*, 2006).

However, there are some clear drawbacks of this method as many peaks of the chromatogram might not represent a single compound but are result of co-elution of multiple constituents. This is one of the possible reasons because the UV spectrum of some peak did not correspond to any flavonoids in the software database such as the one relative to formononetin (see Tab.7).

The chromatograms relative to the sample C19II9 and both the acid- and the basic hydrolysis are reported in fig. 12, in the picture glycosides- and the aglycones part are highlighted.
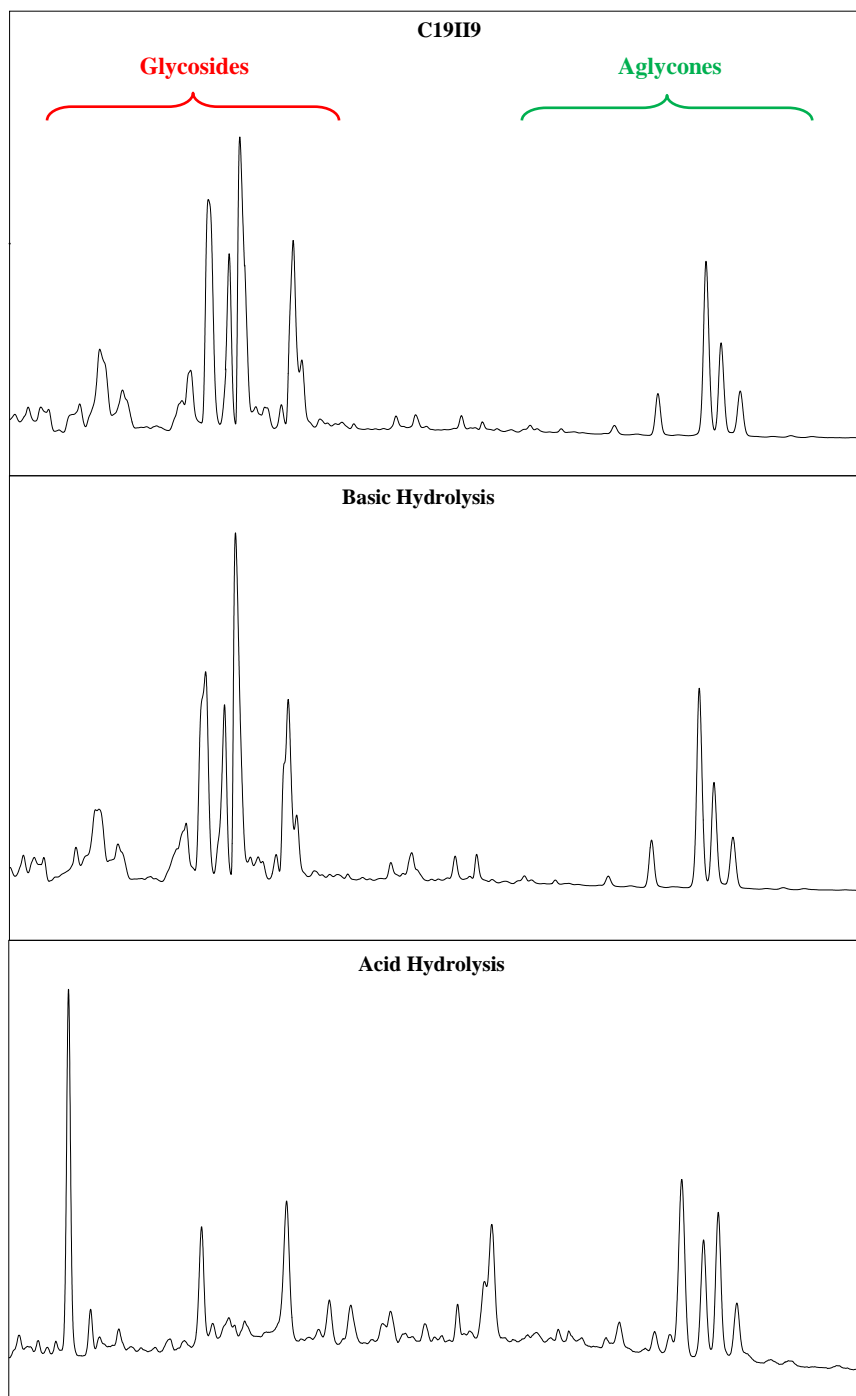

Figure 11 Chromatograms of sample C19II9 up: extract. Center: basic hydrolysis, low: acid hydrolysis

We are aware that advanced HPLC-MS methods using advanced high resolution detectors are more suitable for metabolomics and profiling studies, however, these techniques were not available to us for this study.

The HPLC-MS analysis was carried out both the positive ion mode and the negative ion mode. Comparing the two chromatograms obtained in different modes, we must say that the in the positive ion mode the intensity of the peaks was higher than in the negative one. Moreover, the chromatogram profile was more similar to the HPLC's profile. Furthermore, previous studies show that in the positive ion mode, the spectra of molecules characterized by the presence of a sugar moieties contains significantly more structural information than observed in the negative ion mode (Huhman and Sumner, 2002).
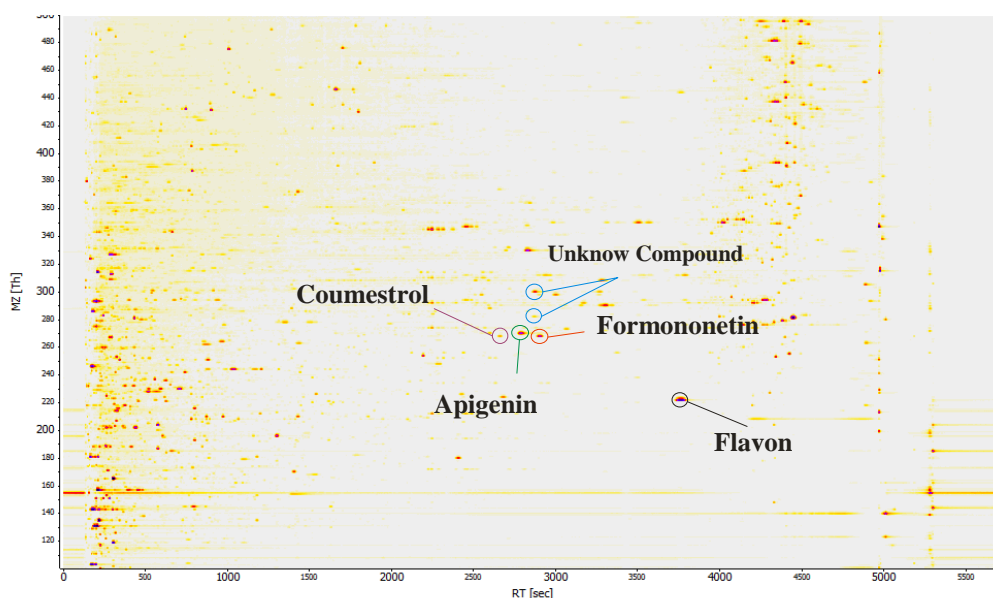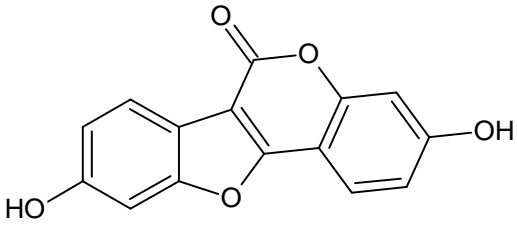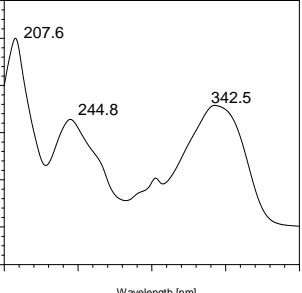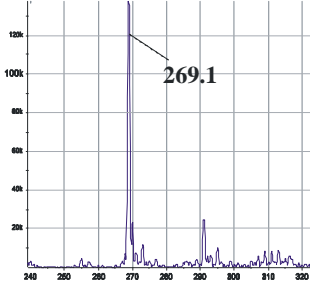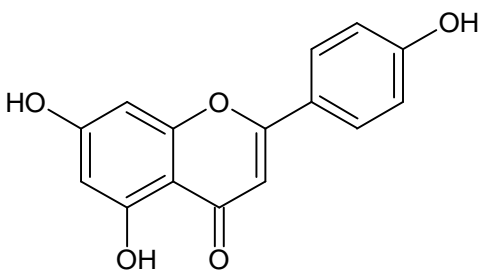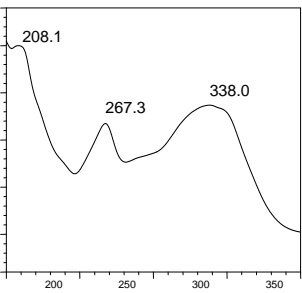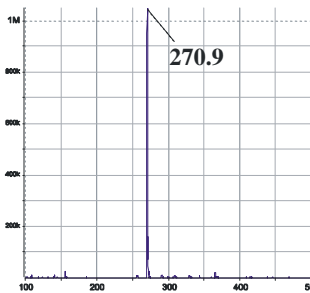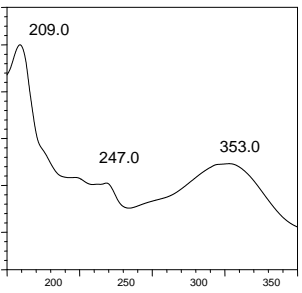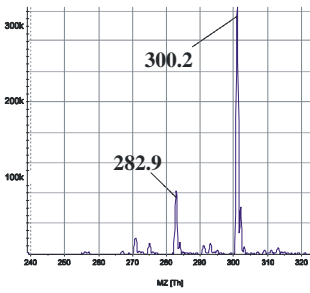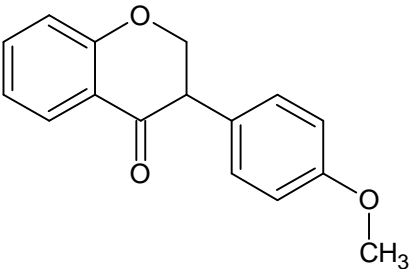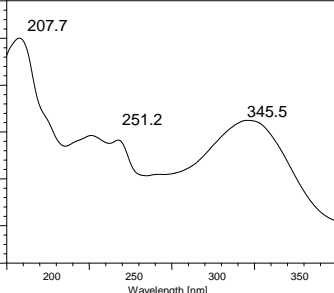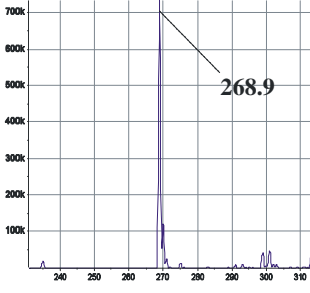

Figure 13 TIC chromatogram from LC-MS of sample B25II9

Fig.13 shows the results of the HPLC-MS analysis in positive ionization mode, using the same conditions as for HPLC-DAD. The horizontal axis reports the retention time in seconds while in the vertical axis shows the m/z ratio. Since the analysis was carried out in the positive ion mode, the peaks in the chromatogram are relative to the molecule with a extra proton added $[M+H]^+$ so the m/z value is relative to their molecular weight plus one.

By matching the chromatograms from HPLC-DAD with those from HPLC-MS obtained under same conditions, we were able to partially identify some of the flavonoids, isoflavonoids aglycones and the internal standard which are highlighted by the circles. The spots highlighted in Fig. 13 are relative to coumestrol, apigenin, an unknown compound and the formononetin and can be found in the HPLC-DAD at similar time. Their identity was thus confirmed by both UV- and MS spectra. These are reported in Table 7, including also the spectra of the unidentified aglycones.

Table 7 Identification of the four peaks highlighted in Fig.13.

Compound	UV-Spectrum	MS-Spectrum
Coumestrol		
	207.6 / 244.8 / 342.5	269.1
Apigenin		
	208.1 / 267.3 / 338.0	270.9
Unknown Compound		
This peak is the result of the co-elution of two compounds. One of them with the molecular weight of 282 g/mol and the other one of 299 g/mol. Since there are not known aglycones with this MW the probably are the derivates of fragmentation of macromolecules.	209.0 / 247.0 / 353.0	300.2 / 282.9
Formononetin		
	207.7 / 251.2 / 345.5	268.9

After the major compounds of the extract have been identified and quantified, the data were subjected to a statistical analysis.

The hypothesis is that different varieties of alfalfa are characterized by a different content in phytoestrogens and the phytoestrogens content is also related to the amount of stem density and stem weight and so, this parameter might is related to the fiber matrix and if isoflavonoids are present in the fibrous matrix, this might be an easy marker for the agricultural practice. Through the multifactorial statistical analysis of the collected data was possible to find the relationship between the content of phytoestrogens, genetic background and stem features.

Two statistical analyses have been performed for the statistical analysis of the data from HPLC-DAD profiles, including two unidentified glycosides peaks and four aglycones including apigenin and coumestrol: Redundancy analysis (RDA) and the analysis of the principal components (PCA).

RDA is statistical correlation analysis which allows the derivation of a specified number of synthetic variables from one set of (independent) variables that explain as much variance as possible in another (independent) set. It is a multivariate analogue of regression. Thus in this case this kind of analysis is perfectly appropriate in order to explain the variance of the flavonoids content related to the variety or the stem's features.

PCA transforms a set of observations of possibly correlate variables by an orthogonal transformation into principal components which are a set of linearly uncorrelated variables. The advantage is the simplification of the statistical analysis which is represented by the reduction of the parameters to considerate because the principal components are less than the original variables.

Table 8 Results of redundancy (RDA) and principal component analyses (PCA) investigating effect of explanatory variables on isoflavones profile of alfalfa forage.

Analysis	Tested variables	Explanatory variables	Covariate	% axis1 (all)	F 1 (all)	P 1 (all)
RDA	F	variety	-	16.6 (28.0)		0.496 (0.722)
		stand	-	8.3 (14.1)		0.300 (0.236)
		variety	stand	15.3 (26.7)		0.598 (0.774)
PCA	F	-		50.1		
			PC2	(18.1)		

% axis1 (all) – variability of abundance or biomass explained by canonical axis 1 or by all axes in brackets; F 1 (all) – F statistics for the test of axis 1 or all axes in brackets; P 1 (all) – permutations) for the test of axis 1 or all axes in brackets.

The results after analyzing the peak profile of the HPLC-DAD chromatogram using the PCA analysis are shown in Fig.14. PC1 and PC2 explained a high percentage of the total variance. PC1 explained the 50.1% of the total variance while PC2 the 18.1%.As shown in Tab.8.

The complete variance in PC2 is mainly defined by coumestrol-and unknown glycoside Gly1-content which shown major negative and positive values, respectively. Although, PC2 is mostly connected with coumestrol, which exhibited a negative value, and Gly1-content, in the positive PC2 axis it is also related with the stem features. AW1 and SD were the main stem features contributors of total variance for PC2.

Positive values of PC2 are related to the AW1 (average of weight of one stem), DMY (dry matter yield), MSL (maximum stem length) while the negative value are related to S to 20 (stem long less than 20cm), SD (stem density), sover20 (stem longer than 20cm) and content of coumestrol. Thus, the coumestrol content seems to be connected to stem feature such as SD and S over20.
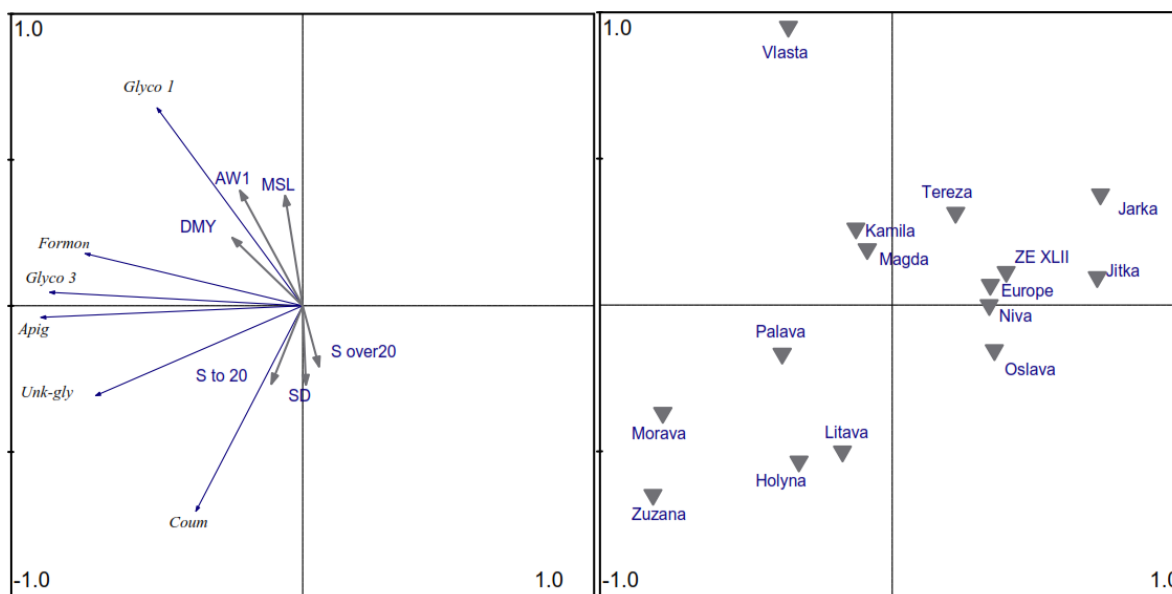
Figure 14 Graphic representation of the PCA analysis.

In the reported scatter plot of the score value of all the genotypes projected to PC1 and PC2, the concentration of the evaluated compounds in cultivars increases proceeding from positive to negative values of PC1. The total variance in PC1 is defined by apigenin content and S over 20, which shown negative and positive values, respectively. Thus, these parameters are negatively correlated.

Considering the influence of the stem's feature on the isoflavonoids content, it increases following the yield weight. However, coumestrol has an autonomous behaviour and its content increases mostly according with the stem density.

Moreover, this means that there is a strong correlation among the isoflavonoid concentrations, which is due to the common biosynthetic pathway that produces almost all the compounds indistinctly. However, the correlation between some of the singles isoflavonoids is different. Formononetin and the unknown glycoside 3 are in a very strict relationship while coumestrol and the unknown glycoside 1 are more independent one from each other.

Negative PC1 axis is highly connected with almost all the considerate compound as well as dry matter yield.

These findings led us to assert that alfalfa plants characterized by a high dry matter yield are rich in the all evaluated compounds. In plant tissues, the phenolics are known to be associated with dietary fibre . PS Lotke Indeed, this association influences both the qualitative and quantitative analyses. This finding led us to assert that cultivars which are rich in dietary fibre might be higher in phenolics, however, nothing is known about their bioavailability and this might be decreased under the fibre content.

Dietary isfolavoniods are almost ubiquitous in plant apparatuses. Their levels in plants vary greatly even between cultivars of the same species and their chemical form is largely influenced by factors such as germination, ripening, processing, or storage. This has been confirmed by our quantitative analyses of coumestrol and apigenin as both the compounds were varying enormously without any apparent influence of the environment or any other known feature (Fig. 9).

According to the scatter plot findings above reported, the varieties located in the negative square for both PC and PC2 are supposedly characterized by high content of all the compounds, coumestrol included, and by a major amount of stems shorter than 20 cm.

Thus, Morava and Zuzana genotypes are supporting isoflavonoids production and accumulation as well as Litava, Holyna and Palava. Thus, these varieties fit with the pharmaceutical purposes reported above, instead, they should be avoided in feeding sheep with fertility problems. A different situation occurs in the cultivar Jarka, which is located positive plot of both PC1 and PC2 variables, is characterized by the lowest content of both apigenin and coumestrol. The variety Vlasta is located in the plot relative to positive value of PC2 and negative of PC1 and is characterized by a high content of apigenin and a low content of coumestrol, according with the above reported findings.

However, most of the relationships described above are rather of a low significance and might be interpreted with caution.

In order to explore underlying trends in the dataset, the distribution of the selected compounds concentration was investigated by redundancy analysis (RDA) considering the influence of the cultivar genetic background and the stems features.

The RDA indicates that relative ratios of investigates compounds differs between stems features such as stem density (SD), dry matter yield (DMY), average of one tem weight (AW1), stem shorter than 20cm (sto20), stem longer than 20 cm (Sover20). Results shown in Fig.15.

Considering the variation selected compound contents, the stems features interaction was not significant (p=0.3). Although, there was a trend in the graph showing that the horizontal axis of the graph is mostly related to the differences in the content of all the evaluated compounds. There was some association along the positive vertical axis with the stems over20 cm and the stem density.
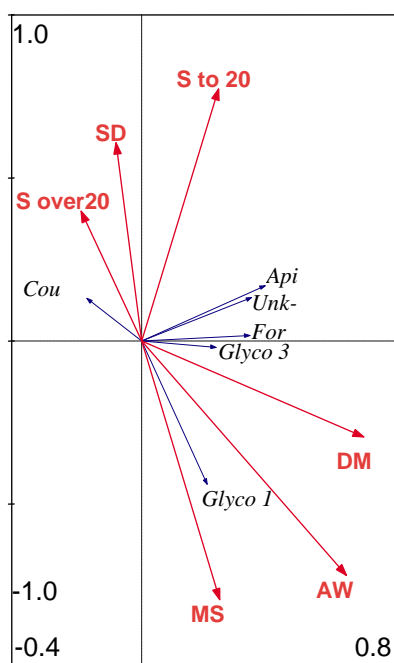

Figure 15 Graphic representation of redundancy analysis of the content of the evaluated compounds among the evaluated alfalfa varieties correlated to the stems characteristics.

While, increasing concentration of Glyco1, dry matter yield and the average weight of one stem was spread along the positive horizontal axis, generally, coumestrol content, sover20 and SD were opposite. As well as coumestrol, sover20- and SD-vector are strictly associated with negative horizontal axis. This is hypothetically due to a correlation between the coumestrol content and these characteristics. The correlation between coumestrol concentration and SD is evaluated by a follow-up statistical analysis.

Since the concentration of coumestrol seemed to be related to Sto20 from PCA, while it seems to be related to Sover20 in the RDA, we conclude that there is no influence between the stem length and the coumestrol content. The coumestrol independent behaviour is confirmed also by this analysis because its vector is related to the negative values of the axis while all the other vectors are going through the side of the same axis.

This is thought to be due to the independent biosynthetic pathway of coumestrol, which is autonomous from the isoflavonoids one. Despite the coumestrol exception, there were strong associations between all compound contents along the positive the horizontal axis.

From the obtained results of the RDA, we assert a positive influence of factors such as maximum stem length (MSL), DMY and AW1 on the concentration of all the measured compounds except for coumestrol which is negatively correlated with the average of the weight of one stem. All these parameter linked to selected compounds concentrations are related to the plant fibre. This finding has been already discussed above.

The different direction of the coumestrol- and the Gly1 vectors mean a possible negative correlation between the contents of these two compounds. It might be hypothesized, that these components mighht be somehow related. Such as, it is known that plants during the abiotic stress release isoflavone aglycons from the glycosidic bonds (Kessmann *et al.*, 1990). The compound A might be theoretically a coumestrol glysocide (although this has not been

confirmed by the DAD spectrum that was not similar neither by the LC-MS analysis) or its unknown glycosidic precursor.

Second RDA was carried out in order to better understand the influence of cultivar on the evaluated concentrations (Fig.16). The cultivar influence on the content of the selected compound is significant as well and it explained 28.0% of the concentration variability, which is almost twice higher than the influence of the stem features. According with the graphic explanation of this RDA analysis, the cultivars are located in the plot following the growing content of the evaluated compounds proceeding from negative value of the horizontal axis to the positive. The total variance in the horizontal axis is defined by the cultivars Morava and Zuzana in the positive side and Jarka and Jitka in the negative side.

This confirmed the results obtained from the PCA which found Zuzana and Morava genotypes as the ones enhancing the production of the selected compounds. Varieties Jarka and Jitka showed the opposite proprieties.
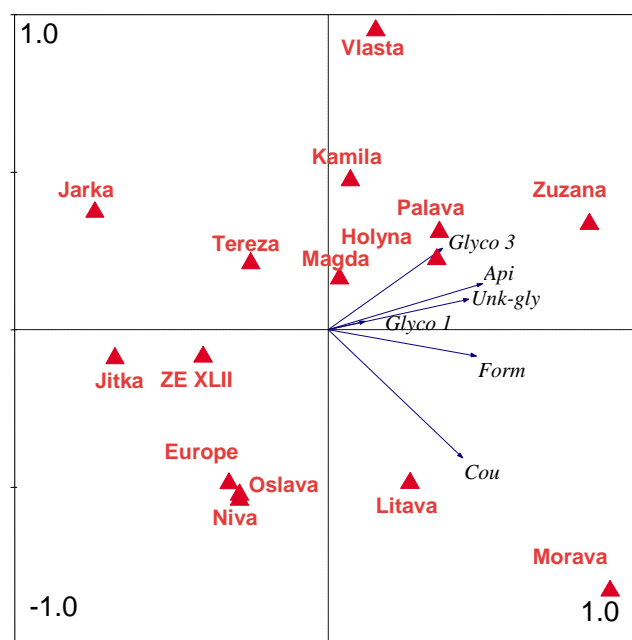

Figure 16 Graphic representation of the Redundancy analysis of the concentration of the evaluated compounds among the alfalfa varieties.

Thus, Jitka and Jarka are the varieties characterized by the lowest content of all the compounds, while, Morava and Zuzana are the ones with the higher contents. According with the above reported findings, the varieties Jitka and Jarka are suitable for fodder purposes in case of feeding sheep with fertility problem in order to decrease the risk related to the isoflavonoids intake. Consequently, in the latter case, varieties characterized by an high dry matter yield should be avoided.

Moreover, the vertical axis of the scatter plot defines the differences in concentration of coumestrol and Gly1, negative- and positive-values, respectively. This confirms the results obtained from the previous RDA based on the stem features.

As shown in Fig.17 there was a negligible correlation between coumestrol and apigenin content among the cultivars. However grouping the samples according to the origin field, their productions seem to be differentially enhanced as it is shown in Fig.18.
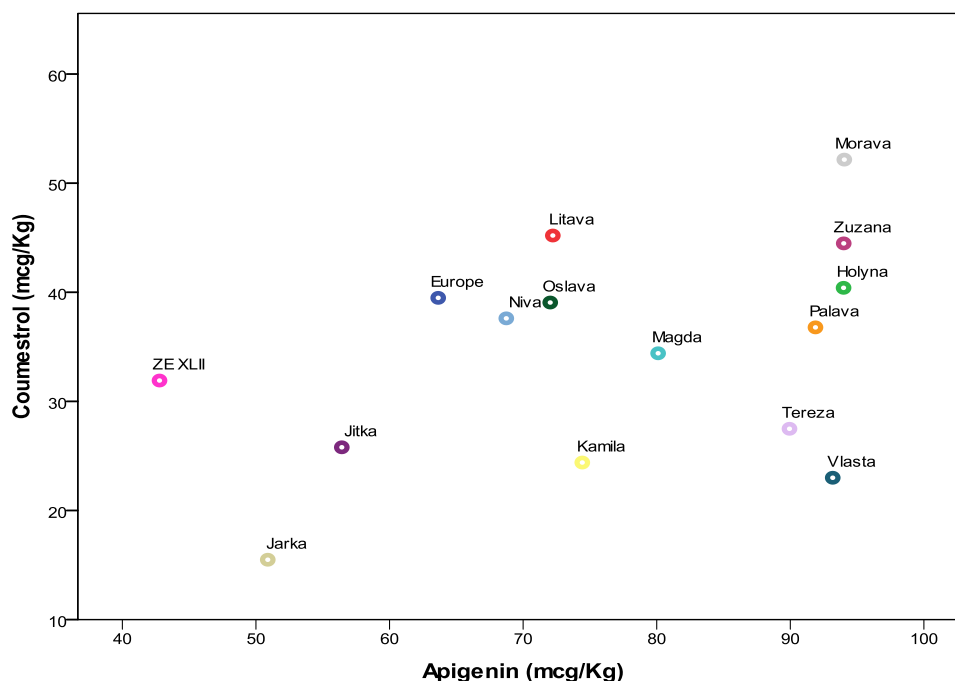

Figure 17 Correlation between coumestrol and apigenin contents among the considered varieties.
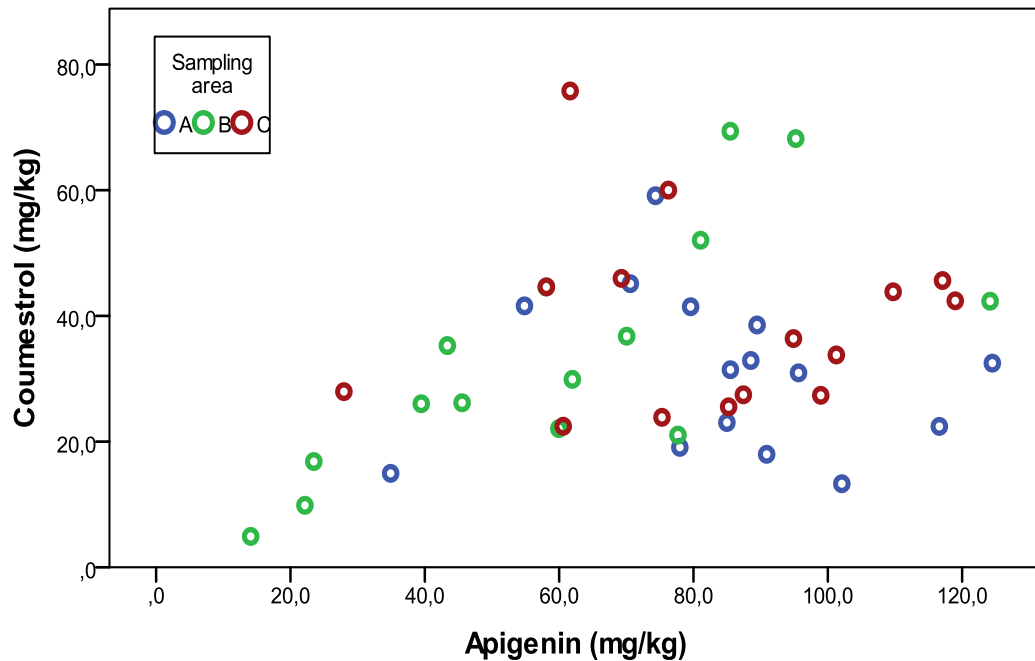
Figure 18 Correlation between coumestrol and apigenin contents, samples are grouped for origin field.

Field-A and -C appear to slightly promote the production of apigenin at the expense of the coumestrol. Despite, field B supports a linear relationship occurring between the concentrations of the two analyzed compounds. However, there is not enough data for a clear statement in this.

Both the RDA and PCA have shown a correlation between coumestrol content and stem density, thus a statistical correlation analysis was carried out. The results of this analysis showed a negligible correlation with a correlation index of 0.137. A graphic explanation of the results is shown in Fig.19. Such a low correlation between the considerate factors may be due small number of samples analyzed. So, we assert that in the samples in our possession there were no significant correlations between coumestrol content and stem density of the alfalfa plants, although further studies are required.

Although a negative correlation between coumestrol content and AW1 was shown in both the PCA and RDA, the correlation analysis between these two factors showed a correlation index of 0.184 resulted which indicates a

negligible relationship. A larger study is probably necessary in order to find out the real influence of the average of one stem on coumestrol content.
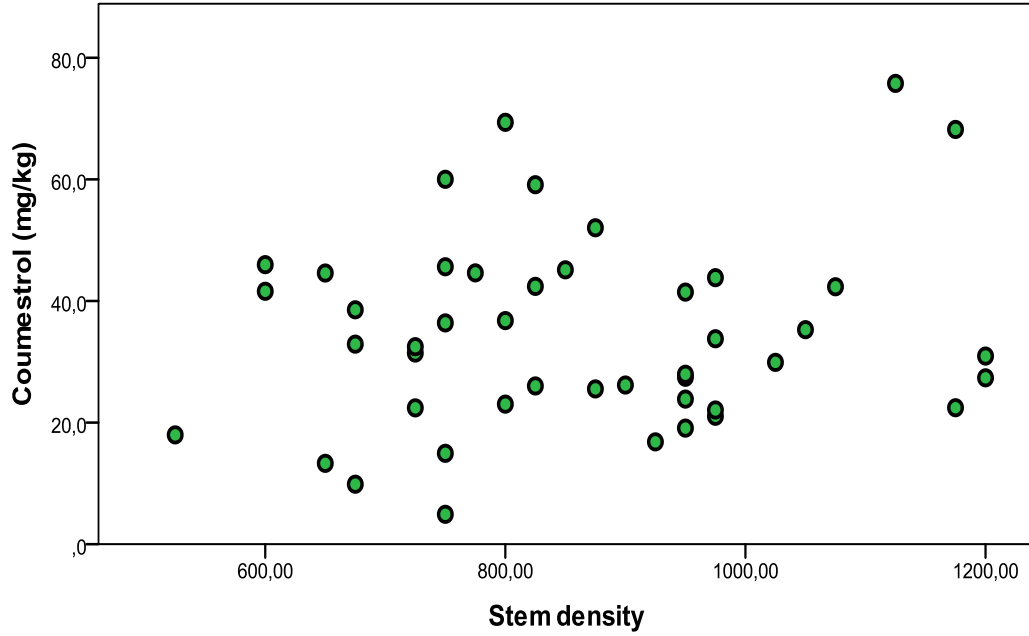

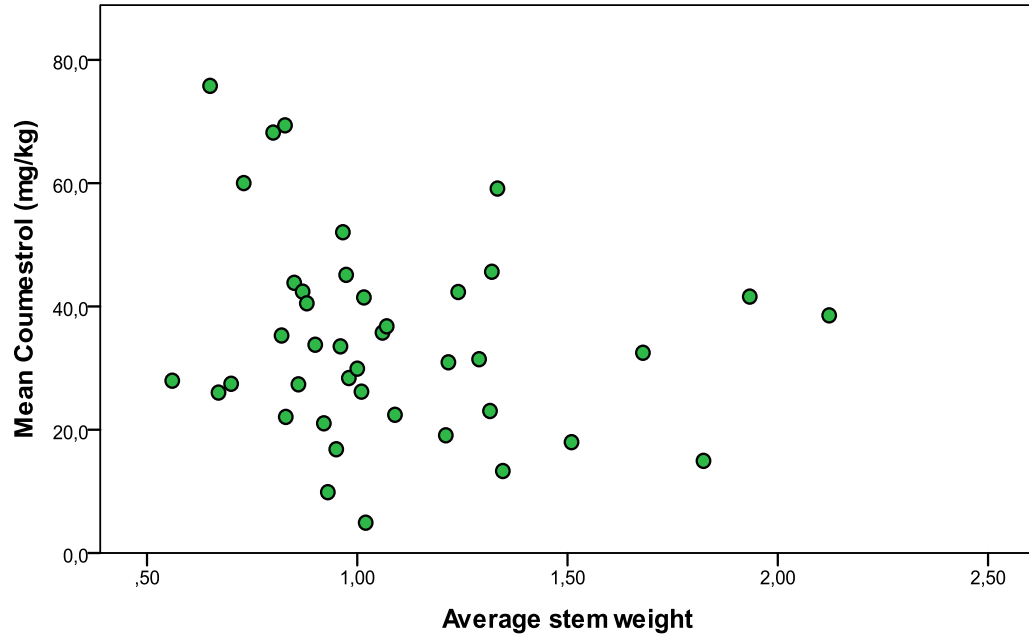**Figure 19 Correlation of coumestrol content and stem density(number of stems/m^2).**

Figure 20 Correlation between coumestrol content and the average of stems weight.

6 Conclusion

According to our knowledge, this study is the first report on phytoestrogens contents in Czech alfalfa cultivars. The analytical HPLC-DAD quantification method developed within this thesis may be useful for routine identification of major flavonoids apigenin and isoflavonoid coumestrol in alfalfa but possibly even in other legumes. Moreover, the results from the LC-MS analysis gave a better insight in almost all the phenolic compounds present in alfalfa sample.

The quantities of coumestrol and apigenin obtained on fifteen Czech cultivars from the second cut of 2009 varied considerably with the genotype of the cultivars.

This great variation presents a challenge in alfalfa production and breeding it for different uses such as for feeding animal or for human nutrition according with the phytoestrogens content.

In particular, this work demonstrates that a genetic improvement program may be conducted in order to create new alfalfa varieties by selecting the cultivars evaluated according with the final purpose. For instance, cultivar Morava and Zuzana, which are rich in both apigenin and coumestrol and thus have a potential of further increasing their contents for food- or pharmaceutical purposes such as developments of food supplements with possible application in prevention of hormone-related disorders e.g. in menopausal age women.

On the other hand, Jarka and Jitka varieties showed relatively lower content independent on the plot location, which may have a potential for e production of new varieties low in phytoestrogens for feeding cattle hords at risk of fertility-related issues.

Although the PCA analyses suggest some relationship between stem features, this is not very clear and the simple correlation shows rather mixed

results and low correlation index. We believe that our results might be of interest for breeders and alfalfa growers.

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