Évelő növényi zöld biomassza, mint értékes fehérje és fitonutrines forrás

Green biomass of perennial crops as valuable source of protein and phytonutrients

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Absztrakt

Az élelmiszer és takarmány előállításhoz szükséges hosszútávú, kiszámítható fehérjebázis biztosítása a mezőgazdaság egyik nagy kihívása hazánkban és más EU-országokban egyaránt. Figyelembe véve az élelmiszer-, takarmányfehérjék gazdasági, etikai, környezeti és ökológiai hatásait, sürgető az alternatív források és technológiák keresése a szója mellett. Az egyik lehetőség a zöld növényi biomassza. Magyarország önellátó lucernából és fűfélékből egyaránt. A lucerna nem csupán jelentős fehérjetartalma miatt érdekes, hanem esszenciális zsírsavak és értékes fitonutriensek forrása egyúttal. Ezzel együtt a fitonutriensek minőségi és mennyiségi összetétele nagyban függ a zöld biomassza feldolgozásától. A nedves frakcionálás során keletkező zöldléből többféle koagulációs technikával értékes levél fehérje koncentrátum (LFK-t) állítható elő. A folyamat elméleti alapját a zöld biofinomítás fogalma foglalja magában.
Munkánk során a frakcionált lucerna zöld biomassza fitokémiai összetételét vizsgáltuk, különös tekintettel az LFK frakcióra, mint közvetlenül felhasználható, fehérjében gazdag (40 – 45 m/m% N) takarmány-, táplálékforrásra. A lucerna frakciókban található fitonutriensek UHPLC-ESI-MS kapcsolt rendszerben kerültek azonosításra. A fenolos komponenseken belül a flavon vázas vegyületek domináltak, jellemzően glikozidok formájában. Hidroxilált metoxiflavont a zöldlé és rost frakciókban találtunk. Izoflavonok közül ononint, alfalont és formonetint mutattunk ki. Kilenc szaponint és hét ismeretlen szaponin aglikont sikerült azonosítani.

kulcsszavak: növényi fehérje, LFK, zöld biofinomító, fitonutrinesek
JEL kód: B01

Abstract

Long-term predictable and reliable protein supply for food and feed production is one of the great challenges of today's agriculture both in Hungary and other EU countries. Considering economical, ethical, environmental and ecological aspects of food/feed proteins it is urgent to find alternative sources with technologies besides soy. Among alternative plantbased protein the green biomass has gained an intensive attention. Hungary is self-sufficient in alfalfa and grasses as dedicated green protein source. At the same time green biomass of alfalfa is more than only protein source, it is a rich repository of essential fatty acids and phytonutrients. The quality and quantity of phytochemicals partially depending on the processing of green biomass. After wet fractionation leaf protein concentrate (LPC) as potenital final product can be obtained from the green juice fraction using any coagulation method. The theoretical basis of this process is included in the concept of green biorefinery.
In our work the phytochemical composition of fractionated alfalfa green biomass was evaluated, with special regard to LPC fraction as directly exploitable, protein-rich (40 – 45 m/m% N) feed/food source. UHPLC-ESI-MS was applied to identify different phytonutrients. The phenolic compounds were significant part of the identified compounds. Among flavonoids

flavones were dominant mostly in the forms of glucosides. Just few flavone aglycones could be identified such as apigenin and luteolin. Hydroxylated methoxyflavone could be isolated from green juice and fiber fractions. Three isoflavones were found such as ononin; alfalone and formonetin. Nine saponins could be identified and seven unknown saponin aglcyons.

keywords: plantbased protein, LPC, green biorefinery, phytonutrients

Introduction

Adequate quantity and proper quality of protein supply is challenging from the aspect of both increasing world population and increasing affluence of the emerging economies (Pyett et al. 2019). According to FAO prediction, global meat production is projected to be increased by 16% from 2015 to 2025. However, the growing demand for animal-based foods are creating severe problems of persistent economical (as concern the import exposure) environmental as well as social degradation. These problems are further exacerbated and affected by climate change, biodiversity loss, water stress, land degradation, and water pollution (Henchion et al. 2017). Due to the strict regulation of the protein source of feed in the EU and the spread of environmentally and health-conscious consumer habits, plants are playing an increasingly important role in protein supply.

In terms of plant organs, the protein accumulates mainly in the leaves and fruits, including seeds. Currently the feed and food industry relies primarily on seed-based protein. Soy protein accounted for the largest share of the overall plant proteins market in 2019. However locally produced green leafy shoot originated protein as alternative protein source could support feed safety and computability. In addition to protein, the biological active compounds such as phenolics, polyunsaturated fatty acids, vitamins, photosynthetic pigments confirm the relevance and worth of green biomass. Depending on the plant species, the phytonutrients composition of green biomass is varied. For instance, forty-six polyphenolic compounds include phenolic acids, flavones flavonols, flavan-3-ols and stilbenes as well as several carotenoids, chlorophylls and triterpenoids have been revealed in leaves of *Fallopia japonica* and *Fallopia sachalinensis* (Lachowicz et al., 2019). Four isoflavones (biochanin A, daidzein, formononetin, and genistein), coumestrol, condensed tannins and triterpene saponins were analyzed from leaves of perennial legumes including clovers (Trifolium pratense L. and T. medium L.), medics (Medicago sativa L. and M. lupulina L.), sainfoin (Onobrychis viciifolia Scop.) and milkvetches (Astragalus glycyphyllos L. and A. cicer L.) in varied concentrations (Butkute et al., 2018).

With sustainability in mind, the green biorefinery concept uses the biological building blocks of locally produced, fractionated green biomass to produce as high as value-added products for feed/food and other industrial purposes.

Following the green fractionation process combined with the microwave coagulation method (based on our patent), the aim of the present work is to qualitatively analyse the phytochemical composition of alfalfa (*Medicago sativa* L.) green biomass fractions / products.

Materials and methods

Experimental installation

A small plot experiment was conducted in 2017 at the Horticultural Demonstration garden of the University of Debrecen, Hungary (47°33'N; 21°36'E). *Medicago sativa* 'Tápiószelei' variety was included into the whole experimental work.

The experiment was set up in a randomized complete block design with 3 replicates, experimental plot was 2x4 m between pots were 0.4 m space.

Processing of green biomass

For sampling 1-1 kilogram of green leafy shoots were mechanically pressed and pulped by a twin-screw juicer (Angel Juicer 7500, Busan, South Korea) into fiber and green juice fractions. Later, the green juice was coagulated used by microwave assisted thermal coagulation at 80 ◦C based on Fári and Domokos-Szabolcsy (2019) patent. After this process the coagulated protein fraction (leaf protein concentrate = LPC) was separated from brown juice using moistened 100% natural unbleached cotton cloth filter (pore size = 10 microns). Fractionation and coagulation were performed in three replicates.

The green biomass originated fractions such as green fiber, green juice and LPC were lyophilized using the Alpha 1-4 LSC Christ lyophilizer, after powdering dry samples were stored in -20 C^o, dark place for further analysis. The brown juice was keep as liquid in -20 C^o too.

Total phenolic content

The total phenolic content of green biomass originated fractions were determined spectrophotometrically by the Folin-Ciocalteu method according to the Singleton & Rossi (1965) with some modifications.

Qualitative analysis of phytochemicals in alfalfa green biomass originated fractions by UHPLC-ESI-MS

Hydro-alcoholic extracts were prepared from each fractions (green juice, fiber, LPC, brown using methanol:water solution at ratio of 7:3. The mixture was stirred on 150 rpm for 2h at room temperature. The hydro-alcoholic extracts were filtered using 0.22 μm PTFE syringe filter.

The phytochemical analyses were performed by UHPLC-ESI-MS (Ultra-High Performance Liquid Chromatography-electrospray ionization/mass spectrometry) technique using a Dionex Ultimate 3000RS UHPLC system (Thermo Fisher, USA) coupled to a Thermo Q Exactive Orbitrap hybrid mass spectrometer equipped with a Thermo Accucore C18 analytical column (2.1 mm × 100 mm, 2.6 μm particle size). The flow rate was maintained at 0.2 mL/min. The column oven temperature was set to 25°C ±1 °C. The mobile phase consisted of methanol (A) and water (B) (both were acidified with 0.1% formic acid). The gradient program was as follows: 0 - 3 min, 95 % B; 3 - 43 min, 0 % B; 43 - 61 min, 0% B; 61 - 62 min, 95% B; 62 - 70 min, 95% B. The injection volume was 2 μL.

Mass spectrometry (MS) conditions were as follow, Thermo Q Exactive Orbitrap hybrid mass spectrometer (Thermo Fisher, USA) was equipped with an electrospray ionization (ESI) source. The samples were measured in positive and negative ionization mode separately. Capillary temperature: 320 °C. Spray voltage: 4.0 kV in positive ionization mode and 3.8 kV in negative ionization mode, respectively. The difference between measured and calculated molecular ion masses was less than 5 ppm in every case. The data were acquired and processed using Thermo Trace Finder 2.1 software based on own and internet databases (Metlin, Mass Bank of North America, m/z Cloud). After processing the results were manually checked using Thermo Xcalibur 4.0 software. The compounds found in the extracts were identified on the basis our previous published works or data found in literature using exact molecular mass, isotopic pattern, characteristic fragment ions and retention time.

Results

After processing, the green biomass originated fractions are presented on the Fig. 1. The quantitative distribution of fractions were the follow: leaf protein concentrate (LPC)16,3 % green fibre fraction 27,9%. brown juice 40,3 %. Because of the laboratory condition the process was discontinuous therefore we need to calculate with ~15,5% accumulate loss. However, in a continuous industrial scale the accumulated loss is negligible.
The average crude protein content of LPC has varied between 47.33 ± 2,00 m/m% DW. Furthermore the green fibre fraction has 13,5 ±1,16 m/m% N % DW, brown juice less than 17.07 m/m% DW crude protein.

Figure 1: Alfalfa green biomass originated fractions: leaf protein concentrate (LPC), green fibre, brown juice Source: Own construction based on Kovács Z. (2020)

Alfalfa LPC is not even concentrate of water soluble proteins of leaves, it enriched with different metabolites, well known, mostly carotenes and xanthophylls (Knuckles et al. 1972). Along with it polyphenols are also considerable. Stochmal and co-workers (2001b) reported more than 20 different flavonoid compound from alfalfa aerial part. Based on our results the average polyphenol content of LPC's were 88.6± 12.2 and 87.5± 7.9 µg/g GAE gallic acid equal.
As concern the phytochemical composition 154 components were identified in fractionated green biomass of Tápiószelei variety. These are sortable as proteinogenic and non-proetinogenic amino acids (22), phenolic acids (10), calcons (2), flavonoids (43), coumarins (1), coumestans (4), saponins (22), fatty acids (8), vitamins and other organic acids and other compounds (13). Figure 2. shows the component distribution between fractions. 39 compounds were detected in all four fraction. These are amino acids, vitamins and most of the flavonoids. Interestingly 22 phytochemicals such as methoxyflavones were found both in the green juice and in the fibre fraction, but after thermal coagulation we couldn't detect in the obtained fractions. Among methoxyflavones, dimethoxy-hydroxyflavone, methoxy-trihydroxyflavone and dimethoxyflavone were identified. These compounds announced as anti-inflammatory, because of the -OCH_3 groups which facilitate the absorption from the intestinal *in vivo* and enhance drug reaction (Balázs 2014). Also, phenolic acids, like salicylic acid-O-hexoside, vanillin and coniferyl aldehyde, hydroxylated fatty acids as well as dihydroxydodecanoic, hydroxy-octadecatrienoic and 2-hydroxyhexadecanoic acids were identified in the fractions which didn't get any thermal treatment.
Most phytochemicals (81) were separated in leaf protein concentrate (LPC) and 21 components of these were found only in this fraction. Characteristically LPC's compounds were pheophytin A and B, chlorogenic acids and several apigenin glucosides.

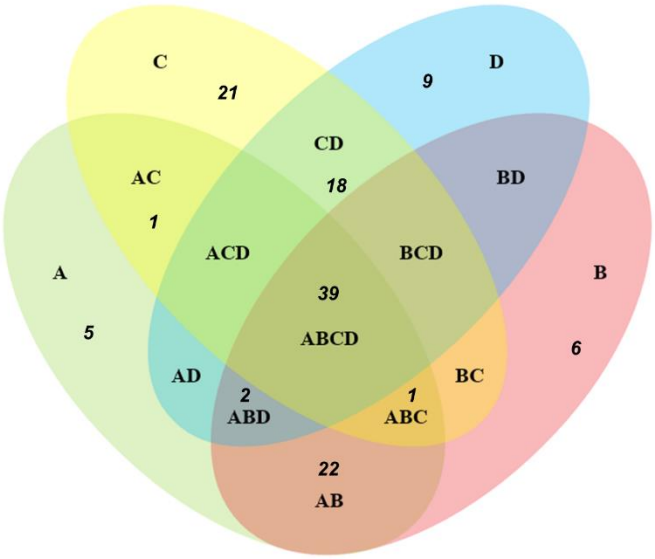

Figure 2: Numerical distribution of identified phytonutrients in alfalfa green biomass originated fractions. A: green juice, B: green fibre, C: LPC, D: brown juice Source: Own construction based on Kovács Z. (2020)

With more than 10000 different structures, flavonoids are one of major group within polyphenolic compounds in plants. Flavonoids are known in general as antioxidant, antihypertensive and antitumor compounds in the human body (Gholami et al. 2014). Table 1. shows the detected flavonoids from the four examined alfalfa green biomass originated fractions. Altogether 43 flavonoids were determined, mainly apigenin and luteolin backbone connected with one or more glucuronide side chains. Other characteristic backbones are tricin, chrysoeriol, naringenin, quercetin and dihydrokaempferol. Among isoflavonoids, formononetin, ononin, alfalon and biochanin A were identified which are known as phytoestrogens. Only formononetin was detected in all four fraction. At the same time brown juice was the only fraction which contained all the identified isoflavonids. Based on the data brown juice, as digested plant serum is a good solvent for isoflavonoids and saponins. Coumestrol, as known alfalfa phytoestrogen was detectable in all fractions. Seguin and co-workers (2004) found that coumestrol and apigenin are the abundant phytoestrogens in alfalfa aerial parts followed by luteolin and quercetin.

Besides flavonoids, saponins were the second biggest group of detected phenolic compounds with 22 identified molecules. 7-7 saponin component was obtained from green juice and fibre fractions, 12 from the brown juice and 17 from the LPC. Medicarpin and medicagenic acid 28-O-[xylosyl-(1→4)-rhamnosyl-(1→2)-arabinosyl] ester was detected saponins from all fractions. A European Food Safety Authority (EFSA) report demonstrate that fresh pressed alfalfa juice contains 2-3 % saponin and the LPC has 0,5-1,4 % (EFSA 2009). Based on this and our observations brown juice has a concentrated saponin content compared with the other fractions. Saponins announced as surfactants and haemolytic agents, however they display fungicidal, antibacterial, antiviral, antitumor and hypolipidemic activities (Tava & Avato 2006).

Table 1: Flavonoids in different alfalfa fractions. Legend: glA-glucuronide, feA-feruloyl

Flavonoids	green juice	fiber	LPC	brown juice	polarity	MW	formula
Luteolin-C-hexoside-O-hexoside	+	-	-	-	[M + H]+	611,161	C27H30O16
Luteolin-7-O-glA	+	+	+	+	[M - H]$^-$	461,072	C21H18O12
Luteolin	+	+	+	+	[M - H]$^-$	285,040	C15H10O6
Luteolin-di-O-glA	-	-	-	+	[M - H]$^-$	637,104	C27H26O18
Luteolin-4'-O-glA-7-O-[feA-(→2)-glA-(1→2)glA]	-	-	-	+	[M - H]$^-$	989,184	C43H42O27
3'-Methoxy-4',5,5',7-tetrahydroxyflavone-7-O-glA	+	+	+	+	[M - H]$^-$	491,083	C22H20O13
Apigenin-O-glA	+	+	-	-	[M - H]$^-$	445,077	C21H18O11
Apigenin	+	+	+	+	[M - H]$^-$	269,045	C15H10O5
Apigenin-4'-O-glA-7-O-[glA-(1→2)-glA]	-	-	+	+	[M - H]$^-$	797,141	C33H34O23
Apigenin-O-glucoside-O-glA	-	-	+	+	[M - H]$^-$	607,130	C27H28O16
Apigenin-7-O-[feA-(→2)-[glA-(1→3)]-glA-(1→2)]glA	-	-	+	+	[M - H]$^-$	973,189	C43H42O26
Apigenin-di-C-pentoside	-	-	+	-	[M + H]$^+$	535,145	C25H26O13
Apigenin-4'-O-glA-7-O-[feA-(→2)-glA-(1→2)-glA]	-	-	+	+	[M - H]$^-$	973,189	C43H42O26
Apigenin-7-O-glA	-	-	+	-	[M - H]$^-$	445,077	C21H18O11
Tricin-7-O-glA	+	+	+	+	[M - H]$^-$	505,098	C23H22O13
Tricin-7-O-[feA-(→2)-glA-(1→2)-glA]	+	+	+	+	[M - H]$^-$	857,178	C39H38O22
Tricin-O-hexoside	+	-	+	-	[M + H]$^+$	493,135	C23H24O12
Tricin	+	+	+	+	[M - H]$^-$	329,066	C17H14O7
Chrysoeriol-7-O-glA	+	+	+	+	[M - H]$^-$	475,088	C22H20O12
Chrysoeriol	+	+	+	+	[M - H]$^-$	299,056	C16H12O6
Chrysoeriol-glAl-glA	-	-	+	-	[M - H]$^-$	651,120	C28H28O18
Chrysoeriol-4',7-di-O-glA	-	-	-	+	[M - H]$^-$	651,120	C28H28O18
Tetrahydroxyflavone-O-hexoside	+	+	-	-	[M - H]$^-$	447,093	C21H20O11
4',7-Dihydroxyflavone	+	+	+	+	[M - H]$^-$	253,050	C15H10O4
Methoxy-tetrahydroxyflavone	+	+	+	+	[M - H]$^-$	315,050	C16H12O7
Dihydroxyflavone isomer	+	-	-	-	[M - H]$^-$	253,050	C15H10O4
Dimethoxy-hydroxyflavone	+	+	-	-	[M + H]$^+$	299,092	C17H14O5
Methoxy-trihydroxyflavone	+	+	-	-	[M - H]$^-$	299,056	C16H12O6
Dihydroxy-dimethoxyflavone	-	+	-	-	[M + H]$^+$	313,071	C17H14O6
Dimethoxyflavone	-	+	-	-	[M + H]$^+$	283,097	C17H14O4
Vicenin-1	-	-	+	-	[M + H]$^+$	565,156	C26H28O14
Vicenin-3	-	-	+	-	[M + H]$^+$	565,156	C26H28O14
Isoquercitrin (Hirsutrin)	+	+	-	-	[M - H]$^-$	463,088	C21H20O12
Quercetin-3,4'-di-O-glucoside	-	-	-	+	[M - H]$^-$	625,140	C27H30O17
Dihydrokaempferol-O-hexoside	+	+	-	-	[M - H]$^-$	449,108	C21H22O11
Naringenin	+	+	+	+	[M - H]$^-$	271,061	C15H12O5
Naringenin-6,8-di-C-glucoside	-	-	+	+	[M - H]$^-$	595,166	C27H32O15
Liquiritigenin	+	+	+	+	[M - H]$^-$	255,066	C15H12O4
Trihydroxyflavanone isomer	+	-	-	-	[M - H]$^-$	271,061	C15H12O5
Formononetin	+	+	+	+	[M + H]$^+$	269,081	C16H12O4
Ononin	-	-	+	+	[M + H]$^+$	431,134	C22H22O9
Alfalone	-	-	+	+	[M - H]$^-$	297,076	C17H14O5
Biochanin A	-	-	-	+	[M - H]$^-$	285,076	C16H12O5
Sum	**24**	**22**	**27**	**26**			

Conclusions

The qualitative analysis of alfalfa (*Medicago sativa* L.) green biomass originated fractions/products was conducted in present work. The different fractions contained flavonoids, polyphenols, vitamins and other secunder metabolites in varied compositions. The leaf protein concentrate which can be direct used as protein source in animal feed mixture contained the highest number of phytochemicals. Among these phytochemicals valuable flavonoids and phenolics were identified. At the same time several isoflavonoids and saponins, known as antinutrients, could also be detected in this fraction however we have no idea the concentration of them. In order to get a complete picture of the biological value and possible utilization of LPC and other fractions, quantitative analysis of the identified compounds is also planned in the near future.

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