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Production Technology, Chemical Composition and Use of Alfalfa Protein-Xanthophyll Concentrate as Dietary Supplement

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Abstract

Modern analyses over the last several decades have shown that alfalfa is an extremely nutrient-rich food source, containing vitamins, minerals and antioxidants. The aim of the study was evaluation of the chemical composition of protein-xanthophyll concentrate from alfalfa (APC) manufactured in France in 2009-2012. Protein-xanthophyll concentrate from alfalfa (APC) was rich in crude protein (534 g kg^{-1} DM), linolenic acid (41.7 g kg^{-1} DM) and minerals, especially calcium (32.9 g kg^{-1} DM) and iron (497.0 mg kg^{-1} DM). Furthermore APC is characterized by low level of crude fiber (5.9 g kg^{-1} DM) and small amount of L-canavanine (3.2 mg kg^{-1} DM). APC may be an important supplement to human diet, because of it required amino acid composition, increased level of linolenic acid ω-3 and high mineral content. The levels of antinutritional compounds do not exceed the acceptable daily limits so the preparation seems to be safe for human health.

Keywords: Protein-xanthophyll concentrate; Nutrients; Fatty acids; Amino acids; Saponins; Functional food

Introduction

Recently an increasing interest has been observed in various dietary supplements that provide specific physiological and prophylactic effects and thus, contribute to improvement of health status [1]. One of such dietary supplements is alfalfa protein-xanthophyll concentrate(APC) (in literature also referred to as protein-xanthophyll concentrate-PX or l'Extrait Foliaire de Luzerne-EFL), produced from alfalfa (*Medicago sativa* L.) and containing over 50% of total protein and 1200-2200 mg/dm^3 of xanthophylls [2-4]. The APC ability to absorb nitrogen, high yielding and interesting nutritive value make it significant from the nutritional perspective. The analyses of the chemical composition showed that the aerial parts of alfalfa, especially leaves, contain-apart from protein and thus valuable amino acids-large amount of provitamin A (β-carotene), vitamins B, C, D, E and K as well as mineral salts: potassium, iron, calcium and phosphorus. Presently, APC concentrate is gaining increasing recognition as a feed additive [5-10]. Ghaly and Alkoaik [11] reported that commercial byproducts of the alfalfa protein concentrate production process have economic values (liquor can be used as bacterial growth medium). Besides, the beneficial composition and a high nutritive value of the preparation promote its inclusion into human diet as a major supplement. Although alfalfa protein-xanthophyll concentrate is a good source for producing functional food, its application is limited due to poor solubility and negative sensory properties [12]. The rich composition of alfalfa is reflected in abundance of secondary metabolites, like e.g. coumarins, isoflavones, naphthoquinone, saponins or alkaloids [13,14]. However, some substances, particularly phytoestrogens (mainly coumesterol) and L-canavanine amino acid, arise concern.

In view of the above, the objective of the research was to determine the chemical composition, in those nutrients, some vitamins and antinutritional factors, in a protein-xanthophyll concentrate from alfalfa (APC) manufactured in France.

Material and Methods

Materials

The research material included samples of a protein-xanthophyll concentrate (APC) from alfalfa (*Medicago sativa* L.) of a Flamand cultivar, from the first harvest at the budding stage (France Luzerne, France) collected for the period of four years (2009-2012). The APC concentrate was produced by a cooperative plant from Planschez, Marne Department, a part of the France Luzerne concern. From each batch of concentrate produced, two averaged final samples were collected four times for analyses during the production process [15].

Production technology

The technology of alfalfa conversion into the protein-xanthophyll concentrate involves pressing process of mechanically disintegrated green matter (Figure 1). The solid fraction (so-called pomace) can be further subjected to drying in a barrel dryer and used as animal feed. The liquid phase (so-called green juice), containing ca. 10-20 % DM undergoes further centrifugal separation. The resultant juice is neutralized to pH 7.5-8.0 with alkaline solution to inhibit the action of phenyloxidases and improve curd structure, then vapor-heated to average temperature of 85-90°C. That facilitates precipitation of a greater quantity of proteins linked with chlorophyll and carotenoid pigments as well as lipids, vitamins and minerals. A coagulate, collected by centrifugation, is dried to a relatively low temperature to prevent damage to protein structures and preserve natural pigments. The heat treatment eliminates bacterial microflora and inactivates thermolabile antinutrients, e.g. trypsin inhibitors [4]. Vitamins and pigments are protected by an antioxidant, in this case-ascorbic acid [16].

Methods

Basic nutrients determination: The samples were studied to estimate a content of basic nutrients according to standard AOAC procedures [17]. Fiber fractions: neutral-detergent (NDF), acidic-

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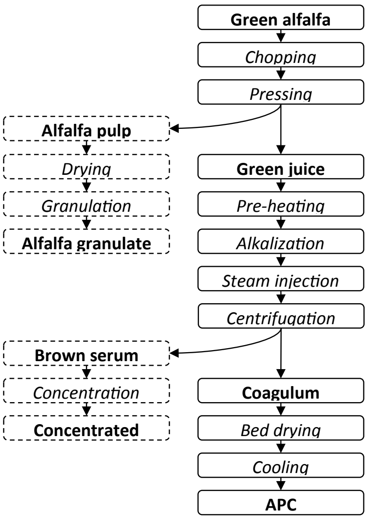

Figure 1: Scheme of alfalfa protein concentrates production technology.

detergent (ADF) and Acidic-Detergent Lignin (ADL), were assayed using the method of Goering and Van Soest [18], whereas cellulose and hemicellulose levels were computed from the difference between NDF, ADF and ADL fractions.

Amino acid determination: Amino acids were determined with ion-exchange chromatography in a 119 Cl Beckman amino acid analyzer. Before the analyses, the samples were acid-hydrolyzed in the presence of 6 M HCl, at 105°C temperature for 24 h. Sulfuric amino acids were established separately after the oxidation [19,20]. Tryptophan was measured following the standard AOAC procedure [17]. The analysis of amino acid composition enabled calculating the Essential Amino Acid Index (EAAI) according to Oser [21].

Fatty acids determination: Methyl esters of fatty acids were determined on a Varian CP 3800 gas chromatograph under the following assay conditions: capillary column CP WAX 52CB DF 0.25 UM, 60 m in length, carrier gas-helium, flow rate-1.4 ml min^{-1}, column temperature-120°C with stepwise increase of 2°C min^{-1} to 210°C, assay time-127 min, injector temperature-160°C, detector temperature-160°C, supporting gases-hydrogen and oxygen.

Mineral determination: A content of minerals, except for phosphorus, was established in an ASA SOLAR 939 UNICAM flame spectrophotometer according to PN–EN ISO 6869 [22], whereas phosphorus level with the spectrometric method acc. to PN–ISO 6491 [23].

β-carotene and vitamin determination: Concentration of β-carotene was measured with the Hager and Mayer-Berthenrath method [24] application. Contents of E and K vitamin were assayed using the HPLC technique with fluorescent detection. Chromatographic separation was conducted with the use of isocratic separation. Quantitative study of vitamin K was conducted at excitation/emission wavelength of 295/330 nm. Estimation of vitamin K and E concentration was based on the parameters of calibration curves plotted for individual vitamins, taking into account the coefficient resulting from the preparation of the experimental material and reference material.

ANFs determination: The content of L-canavanine was analyzed with the ultra-performance liquid chromatography (UPLC), whereas that of coumesterol and saponins-with the high-performance liquid chromatography [25].

Results and Discussion

The basic nutrient levels in the protein-xanthophyll concentrate (APC) from alfalfa from harvests of four subsequent years (2009-2012) were presented in Table 1. A crude protein content reached 533.9 g kg^{-1} on average, whereas slightly lower values (450-470 g kg^{-1}) were reported by other authors [26,27] regarding similar alfalfa preparations but produced under different technological conditions. Apart from a total protein level, its amino acid composition is also vital as it is responsible for the biological value of protein (Table 2). Aspartic acid was shown to predominate amongst amino acids. The sum of exogenous amino acids reached 415 g kg^{-1} protein, with cysteine as a limiting amino acid. It is worthwhile to compare amino acid composition of alfalfa concentrate and that of milk powder. The analyzed APC preparation differed markedly in the content of proline and glutamic acid from milk powder, while the contents of other amino acids were at a similar level (Table 2). The beneficial ratio of amino acids additionally affects their high bioavailability. *In vivo* and *in vitro* study on the digestibility of leaf concentrate proteins demonstrated its elevation up to even 65-85% [28-32]. High digestibility of nutrients of the alfalfa concentrate is the outcome of a low content of crude fiber (5.9 g kg^{-1} on average). The analysis of the concentrate samples taken during four subsequent years showed insignificant differences in the crude fiber content, especially of cellulose and hemicellulose. Excessive dietary fiber, especially its lignin fraction, can reduce nutrient utilization by stable compounds of calcium and iron, whereas a low content of lignins (ADL) in the analyzed concentrate assures good availability of these mineral elements. The preparation from alfalfa is a potent source of minerals (Table 3). Alfalfa is rich in calcium, iron, copper, manganese, phosphorus, potassium, silicon, zinc and many vitamins: A, B_1, B_2, B_3, B_5, B_6, C, D, E, and K [33]. Great quantity of calcium and iron should also be highlighted. Calcium (32.9 g kg^{-1} APC concentrate on average) is indispensable for skeleton development and its supply ought to be increased in the lactation period. Hernández et al. [32] obtained a higher content of this element in the alfalfa concentrate produced upon freezing, however its content in a sample may depend not only on the APC production technology but also on soil and climatic conditions of crop cultivation. Out of the analyzed microelements, the highest level was reported for iron (497.0 mg kg^{-1}). Similar values referring to all mineral elements were presented by other authors [4,16]. Generally, ca. 90 % of absorbed iron takes part in erythropoiesis and importantly, iron from a well-balanced diet prevents iron-deficiency anemia [34]. The results obtained in the study of Vyas et al. [35] demonstrate that alfalfa concentrate is an effective and more palatable alternative to Fe and folic acid supplements for treating anemia in adolescent girls. The intake of a recommended daily dose of the preparation (10 g day^{-1}) covers ca. 30% of daily demand

Nutrients	Mean ± SD
Dry matter	910.4 ± 38.2
Crude ash	102.3 ± 14.2
Ether extract	103.7 ± 14.2
Crude fiber	5.9 ± 1.4
Crude protein	533.9 ± 17.8
Nitrogen-free extract	164.6 ± 10.6
NDF	98.5 ± 6.4
ADF	10.6 ± 1.1
ADL	0.3 ± 0.1
Cellulose	10.3 ± 1.2
Hemicellulose	87.9 ± 6.1

Table 1: Nutrients content in APC (g kg^{-1}) from harvests of four subsequent years (2009-2012).

Amino acids	Protein (mean ± SD)	APC (mean ± SD)
Glycine	51.1 ± 2.1	25.9 ± 1.1
Alanine	65.8 ± 2.4	33.4 ± 1.2
Valine	58.9 ± 1.9	29.8 ± 1.0
Leucine	89.2 ± 3.1	44.9 ± 1.5
Isoleucine	45.8 ± 1.8	23.2 ± 0.9
Methionine	19.8 ± 1.2	10.1 ± 0.6
Cysteine	10.4 ± 1.1	5.3 ± 0.6
Tryptophan	21.4 ± 0.4	11.6 ± 0.2
Proline	43.5 ± 1.5	21.9 ± 0.7
Serine	42.4 ± 6.1	21.8 ± 3.1
Threonine	43.9 ± 1.4	22.1 ± 0.7
Aspartic acid	100.1 ± 7.3	48.7 ± 3.6
Glutamic acid	86.4 ± 4.1	43.6 ± 2.2
Lysine	60.2 ± 2.1	30.5 ± 1.1
Arginine	57.1 ± 1.7	29.3 ± 0.8
Histidine	23.8 ± 1.1	12.1 ± 0.5
Phenylalanine	53.4 ± 1.5	27.2 ± 0.8
Tyrosine	40.4 ± 1.2	20.4 ± 0.6
EAA Index	84.35 ± 3.25	-

Table 2: Amino acids composition (g kg^{-1}) of protein and APC from harvests of four subsequent years (2009-2012).

Macroelements	Average (g kg-1) (mean ± SD)
Calcium	32.9 ± 2.3
Phosphorus	7.9 ± 0.9
Magnesium	1.5 ± 0.2
Potassium	7.4 ± 0.4
Sodium	0.13 ± 0.02
Microelements	Average (mg kg-1) (mean ± SD)
Iron	497.0 ± 56.1
Manganese	81.8 ± 6.2
Copper	10.2 ± 1.4
Zinc	19.4 ± 2.1

Table 3: Minerals content in APC from harvests of four subsequent years (2009-2012).

for calcium and 50% for iron in adult man [16]. The analyzed samples of alfalfa preparation were characterized by a relatively high fat level (103.7 g kg^{-1} on average). Interestingly, the highest contribution to the fatty acid profile was noted for linoloenic acid (ω-3), whereas the total content of polyunsaturated fatty acids reached 60.5 g kg^{-1} (Table 4) and was comparable with that reported in soybean oil and linseed oil [36]. The analysis of the fatty acid profile of APC concentrate fat and fat of linseed and soybean oils demonstrated, however, that the investigated preparation had a higher content of saturated fatty acids and a lower content of monounsaturated fatty acids. In addition, the analyses showed a lower ratio of ω-6 to ω-3 fatty acids which in a human diet should range from 5:1 up to 2:1 [37]. A human body is incapable of synthesizing linoleic acid of the ω-6 group and alpha-linolenic acid of the ω-3 group of fatty acids. These two acids need to be supplied with a diet. It is worth emphasizing that not only their ratio but also their content in diet are of great significance [37]. There is much data pointing to the significance of ω-3 fatty acids in the defense system against the development of cardiovascular diseases and to their anti-inflammatory effects, which may be also important for development of other diseases. Alfalfa contains relatively high quantities of lipophilic vitamins A, D and E (being natural antioxidants) exceeding body demands as well as B-group vitamins and even vitamin K and carotenes which are vitamin A precursors [33]. These vitamins are lost during processing, which has been confirmed in our study (Table 5). The analyzed concentrate was a valuable source of vitamin E and K and of β-carotene. Their average contents in the assayed material reached 428.2; 95.3 and 303.7 mg kg^{-1}, respectively. A daily APC supplementation 10 g covers 100 % of demand for vitamin A and K, and nearly 30 % for vitamin E [16]. Three analyses of each collected sample at a detection limit of 69.3 mg kg^{-1}, did not indicate the presence of coumesterol. Much attention is also given to effects of saponins occurring in alfalfa. The studies on animals (macaques) did not demonstrate any toxic effect of alfalfa saponins [38]. Saponins have been implicated in total cholesterol level reduction and in enhanced secretion of bile, gastric, pancreatic and intestinal juices [39-41]. The studies [42-45] revealed that depending on their chemical structure, they may display antibacterial and antimycotic actions-mainly against selected yeast pathogenic to man. Therefore they may also have immuno stimulatory effects. The highest activity of alfalfa saponin was observed against Gram-positive bacteria, e.g. *Bacillus cereus, Bacillus subtilis, Staphylococcus ureusor Enterococcus faecalis* [14]. The *in vitro* studies demonstrated their efficacy as anti-cancer agents and confirmed their capability to inhibit the development of leukemia cell line in human [46-48]. Contents of individual saponins were presented in Table 6. Their content in green juice from alfalfa (immediately after the pressing phase) ranges between 20 and 30 g kg^{-1}, whereas in the concentrate 5-14 g kg^{-1} [16]. These values are lower than those noted for leguminous plants (30-70 g kg^{-1}) [48]. In the analyzed samples of alfalfa concentrate produced in 2009-2012, the content of saponins oscillated around 10 g kg^{-1}. Another disputable component of alfalfa is L-canavanine amino acid, i.e. a potentially-toxic antimetabolite of L-arginine, that may contribute to the activation of an autoimmune disease (systemic lupus erythematosus). This substance is found in many leguminous plants [49]. Worthy of notice is that its content differs much in particular parts of a plant-the highest level is noted in alfalfa seeds and sprouts (80-150 mg kg^{-1}), whereas in leaves (used for concentrate production) it reaches only ca. 10 mg kg^{-1}. The concentration of L-canavanine in APC is twofold higher than in soybean flour, but significantly lower than in other plant products, i.e. lentil flour or onion. Therefore, diet supplementation with the APC concentrate does not pose any health risk. In the analyzed samples, a mean content of this amino acid was at 3.2 mg kg^{-1}.The analysis of the concentrate samples taken during four subsequent years showed insignificant differences in analyzed components.

Conclusions

Protein-xanthophyll concentrate (APC) from alfalfa is characterized

Fatty acids	FA (mean ± SD)	APC (mean ± SD)
Myristic (C 14:0)	14.4 ± 1.2	1.4 ± 0.2
Palmitic (C 16:0)	176.3 ± 11.2	16.8 ± 1.3
Palmitoleic (16:1)	67.1 ± 5.2	6.4 ± 0.5
Stearic (C 18:0)	28.8 ± 2.1	2.7 ± 0.2
Oleic (C 18:1)	54.9 ± 5.8	5.2 ± 0.5
Linoleic (C 18:2. ω-6)	196.7 ± 15.2	18.8 ± 1.6
Linolenic (C 18:3. ω-3)	437.2 ± 34.2	41.7 ± 3.2
Arachidic (C 20:0)	9.5 ± 0.9	0.9 ± 0.1
Eicosadienoic (C 22:0)	7.9 ± 1.1	0.8 ± 0.1
Tetracosanoic (C 24:0)	7.2 ± 1.1	0.7 ± 0.1
Total	-	95.4 ± 13.3
ΣSFA	244.1 ± 14.5	23.3 ± 1.2
ΣMUFA	122.0 ± 7.2	11.6 ± 0.7
ΣPUFA	633.9 ± 41.3	60.5 ± 4.1
ω-6 / ω-3	4.5 ± 0.2	4.5 ± 0.2

Table 4: Fatty acids composition (g kg^{-1}) of total fatty acids and APC from harvests of four subsequent years (2009-2012).

Vitamins	Mean ± SD
Vitamin E*	428.2 ± 38.9
Vitamin K	95.3 ± 11.1
β-carotene	303.7 ± 34.8
L-canavanine	3.2 ± 0.4
* - as α-tocopherol	

Table 5: Concentration of vitamin E, K, β-carotene and L-canavanine (mg kg^{-1}) in APC from harvests of four subsequent years (2009-2012).

Saponins	Mean ± SD
Zahnic acid tridesmoside	0.97 ± 0.15
Medicagenic acid 3 GlcGlc. 28 AraRhaXyl	0.77 ± 0.14
Medicagenic acid 3 GlcA. 28 AraRhaXyl	5.82 ± 0.43
Medicagenic acid 3 Glc. 28 AraRhaXyl	0.52 ± 0.52
Soyasaponin I	2.30 ± 0.21
Total	10.39 ± 0.82

Table 6: Concentration of saponins (g kg^{-1}) in APC from harvests of four subsequent years (2009-2012).

by a high content of total protein (533.9 g kg^{-1}) and required amino acid composition resembling that of milk proteins, with high contribution of linolenic acid (ω-3) and low dietary fiber, owing to which it may be considered an interesting supplement in human diet. Due to a high content of minerals, calcium and iron in particular, the APC concentrate from alfalfa can be applied in periods of increased demand for these elements or at their dietary deficiency. The content and composition of antinutrients do not exceed the permissible values, and thus the APC preparation seems to be safe for human and animal health. In the case of humans, its dosage should be consistent with the EFSA recommendations. Dietary supplement "Medisat" [50] is produced on the basis of the APC preparation.

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