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SCIENTIFIC OPINION

Opinion on the safety of 'Alfalfa protein concentrate' as food[1]

Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies

(Question No EFSA-Q-2008-031)

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PANEL MEMBERS

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SUMMARY

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to deliver a scientific opinion on the safety of 'Alfalfa protein concentrate' as food.

The novel food ingredient, alfalfa protein concentrate (APC), consists of a complex of protein (45-60 %), minerals (Ca, Fe, Mg), and vitamins (A, D, E, K). APC is extracted from lucerne (*Medicago sativa spp. sativa)* also known as alfalfa. Alfalfa is processed to first provide a press juice from which the proteins associated with carotenoid and chlorophyll pigments are separated by heat treatment and centrifugation and thereafter dried at sufficiently low temperature. The final product is granulated after adding ascorbic acid (600 mg/kg) and stored in inert gas or in cold storage. Alfalfa protein concentrate is currently marketed as animal feed. The applicant proposes the use of APC as a food supplement with a recommended consumption of 10 g per day.

Alfalfa, the source of the protein concentrate, and parts of the plant (seeds, sprouts) have been used for human consumption in the European Community. Thus, alfalfa is a food ingredient that is not considered as a novel food. Alfalfa protein concentrate (dose 10 g/day) has been tested for its nutritional value in several clinical trials carried out in some countries (e.g. Peru, India and Congo). The alfalfa protein concentrate has been used since 1992 as a food supplement to combat malnutrition in several non-EU countries in the world with no reported deleterious effects.

The main concerns of the Members States were the presence of coumestrol, L-canavanine, and ß-carotene in APC and the potential allergenicity of the product.

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Alfalfa foliar extracts contain phytoestrogens (coumestrol and isoflavones) that are known to disturb the reproduction cycle in females. The total isoflavone (daidzein, genistein, glycitein) content of APC is 255 mg/kg, which is similar to the level of total isoflavonoids present in soy products. The suggested consumption of 10 g of APC per day leads to an intake of 2.6 mg of isoflavones which is about 17 % of the average total isoflavone intake via soy-based foods in the French adult population (AFSSA 2005). Also the coumestrol content of APC (78 mg/kg) is comparable to that of the main sources of coumestrol intake, fresh soy sprouts and split peas. The Panel notes that for adults and children weighing over 32 kg, the amounts of coumestrol (0.8 mg) and isoflavones (2.6 mg) in the recommended daily dose of 10 g of APC do not exceed the maximum recommended safe levels of isoflavone consumption of 1 mg/kg body weight per day set by AFFSA (2005). The Panel concludes that the intake level of phytoestrogens in a 10 g daily dose of APC is lower than from other common food sources and therefore does not raise concern.

L-canavanine, which is present mainly in seeds and sprouts of most leguminous plants, has been suspected of being responsible for systemic lupus erythematosus (SLE) activation. However, the L-canavanine concentration of APC (4.3 mg/kg) is very low compared to other common food sources such as lentil flour (2800 mg/kg) and onions (10 000 mg/kg). Moreover, alfalfa protein concentrate did not lead to an anti-DNA_{ds} antibody production (a SLE marker) in a mouse study designed for lupus. The Panel concludes that the L-canavanine content of APC is not of concern.

A daily dose of 10 g of APC can provide up to 7.2 mg ß-carotene. The Panel notes that the use of ß-carotene as a supplement should be regarded cautiously as there may be a very small difference between the levels that may confer health benefits (up to 10 mg/d, mainly from natural sources) and those that may produce adverse effects in smokers in the general population (20 mg/day in the ATBC study) (SCF 2000).

An *in vivo* study showed ingestion of APC to cause slight allergic manifestations in mice sensitized to peanuts. Data are lacking on cross-reactivity with peanut using IgE binding, skin prick testing, or double blind placebo controlled approaches. The Panel concludes that the risk of cross-reactivity in subjects allergic to peanuts cannot be excluded.

The Panel notes that there are no subchronic, chronic, reproduction and developmental toxicological data available on APC. The Panel concludes that other toxicological data concerning levels and effects of anti-nutrients and secondary metabolites (in particular L-canavanine and phytoestrogens) in APC, the information regarding nutritional effects in animal and human studies, and the history of use as a food supplement without reported adverse effects is supportive concerning the safety of APC.

The Panel concludes that the use of APC as a food supplement at the proposed use level of 10 g per day is of no safety concern.

Key words: Alfalfa protein concentrate, lucerne leaf extract**,** coumestrol, L-canavanine, β-carotene, allergenicity

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BACKGROUND AS PROVIDED BY THE COMMISSION

In February 1999, Viridis S.A. submitted a request under Article 4 of the Novel Food Regulation (EC) N° 258/97 to the competent authorities of France for placing on the market 'two leaf extracts from Lucerne' as food.

The competent authorities of France forwarded to the Commission their initial assessment report, which came to the conclusion that an additional assessment was required.

The Commission was informed only in summer 2003. Therefore, on 18 August 2003 the Commission forwarded the summary of the application to Member States.

On 27 February 2004, the Commission forwarded the initial assessment report to the other Member States. Several of the Member States submitted additional comments.

The concerns of a scientific nature raised by the Competent Authorities of Member States can be summarised as follows:

- Presence of coumestrol (a potent phytoestrogen associated with lucerne) in the novel food, especially if the lucerne leaf extracts are used in children's diets. Further toxicological data including reproductive toxicity is needed to evaluate whether the presence of coumestrol and β-carotene in the extracts present any risk for human health.
- Additional toxicological data are needed on the safety of L-canavanine (an amino acid that constitutes 0.1 % lucerne leaf and is an arginine analogue) and other possible immunological agents present. The possibility that systemic lupus erythematosus (SLE)-type auto-immune reactions might be induced by the product should be investigated.
- Potential allergenicity/cross-reactivity with known allergens should be investigated. In particular cross-reactivity between the proteins of lucerne leaf extract and the proteins of leguminous plants causing allergies (such as peanuts) should be assessed. In addition, the amino acid sequences of lucerne proteins should be analysed for potential similarities to the sequences of known allergens.

In consequence, a Community Decision was required under Article 7, paragraph 1 of Regulation (EC) No 258/97.

Since 12 October 2006 the company LRD (Luzerne – Recherche et Développement) was responsible for the application. They prepared a revised dossier limiting the scope of the application to 'Alfalfa protein concentrate'.

TERMS OF REFERENCE AS PROVIDED BY THE COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Food Safety Authority is asked to carry out the additional assessment for 'Alfalfa protein concentrate' as food in the context of Regulation (EC) N° 258/97.

EFSA is asked to carry out the additional assessment, and to consider also the elements of a scientific nature in the comments raised by the other Member States (Annex 3).

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ASSESSMENT

In accordance with the Commission Recommendation 97/618/EC, the alfalfa protein concentrate belongs to class 2 comprising complex novel ingredients which are not, or are derived from sources which have not been, genetically modified. Intact plant, animals, and micro-organisms used as foods as well as food ingredients (e.g. complex carbohydrates, fats, proteins and substances collectively described as "dietary fibre") are included. The assessment of the safety of the ingredient will be based on the data provided by the applicant to comply with the information required for novel foods of class 2.1 (the source has a history of food use in the EU), i.e. the structured schemes I, II, III, IX, X, XI, XII, and XIII.

I. Specification of the novel food (NF)

The proposed novel food ingredient, alfalfa protein concentrate (APC), is extracted from lucerne (*Medicago sativa spp. sativa)*, also known as alfalfa. According to data provided by applicant, APC largely consists of 45 - 60 % proteins, 9 - 11 % fats, 11 - 15 % polysaccharides (insoluble fibre) and 8 - 13 % minerals, and contains also a number of vitamins. The nutritional composition of APC is presented in Table 1. An overview on the test results of a number of analyses for contaminants are summarized in Table 2. The applicant proposes the use of APC as a food supplement with a recommended consumption of 10 g per day. APC can be provided in the form of sachets of powder, tablets or capsules. The Panel notes the high content of PAH exceeding the legal limits.

Alfalfa (lucerne) belongs to the legume family, known for its ability to fix atmospheric nitrogen due to symbiosis between the plant and bacteria which develops in its root system. Two botanical species and their hybrids are classed under the name of alfalfa: *Medicago sativa spp. falcata* and *Medicago sativa spp. sativa*. In France, all the alfalfa populations are *Medicago sativa spp. sativa*. Alfalfa is the most widely grown forage crop in the world, and it is particularly common in hot temperature regions, subtropical regions and at high altitudes. Nearly 32 million hectares of lucerne is grown worldwide. Alfalfa is one of the plants producing the most protein per hectare.

Table 1. **Nutritional composition of APC[(a)]**

Compound (% of APC on raw basis)	**Average (% of APC)**	**Standard deviation**	**Minimum**	**Maximum**	**No of analyses**
Protein	50.8	4.6	45.0	60.0	7
Fat	10.2	0.7	9.0	10.7	8
Polysaccharides (insoluble fibre)	13.3	1.7	11.0	15.0	4
including cellulose	2.5	0.4	2.0	2.9	4
Free carbohydrates (soluble fibre)	1.4	0.5	1.0	2.0	4
Minerals	10.6	1.1	8.1	13.0	12
APC amino-acid profile	**Average (% of APC)**	**Standard deviation**	**Minimum**	**Maximum**	**No of analyses**
Glycine	2.7	0.1	2.5	2.9	6
Alanine	3.1	0.2	2.9	3.4	6
Valine	3.1	0.1	2.9	3.3	10
Leucine	4.7	0.3	4.3	5.5	10
Isoleucine	2.6	0.2	2.4	3.0	10
Methionine	1.0	0.1	0.9	1.1	11
Cysteine	0.5	0.1	0.1	0.6	10
Tryptophan	1.2	0.3	0.4	1.4	8
Proline	2.3	0.2	1.9	2.5	10
Serine	2.1	0.2	1.6	2.3	10
Threonine	2.4	0.1	2.2	2.6	10
Aspartic acid	5.0	0.2	4.6	5.4	10
Glutamic acid	5.6	0.5	5.0	6.9	10
Lysine	3.1	0.2	2.9	3.4	11
Arginine	3.1	0.2	2.8	3.4	10
Histidine	1.2	0.1	1.1	1.3	10
Phenylalanine	3.0	0.1	2.8	3.2	10
Tyrosine	2.2	0.2	1.9	2.5	10
Fatty acids (% of total fat)	**Average (% of total fat)**	**Standard deviation**	**Minimum**	**Maximum**	**No of analyses**
Saturated fatty acids	14.1	1.5	12.1	15.7	5
Palmitic acid	12.3	1.3	10.8	13.8	5
Stearic acid	1.7	0.3	1.3	1.9	5
Monounsaturated fatty acids	1.9	0.5	1.2	2.4	5
Oleic acid	1.9	0.5	1.2	2.4	5
Polyunsaturated fatty acids	46.3	4.7	37.4	54.0	8
Linoleic acid (omega 6)	12.2	0.8	11.2	13.3	8
Alpha Linolenic acid (omega 3)	34.1	4.5	26.2	42.2	8
Omega 6 / omega 3 ratio	0.36	0.05	0.28	0.43	8

Vitamin content (mg/100 g APC on raw basis)	Average	Standard deviation	Minimum	Maximum	No of analyses
A (beta-carotene retinol eq.) (mg/100g of APC on raw basis)	7.674	3.826	3.691	11.991	4
B1	0.219	0.111	0.093	0.300	3
B2	0.443	0.059	0.381	0.500	3
B3	0.426	0.300	0.080	0.600	3
B5	0.000	0.00	0.000	0.000	3
B6	5.790	4.469	0.630	8.400	3
B8	0.015	0.008	0.006	0.020	3
B9	0.134	0.073	0.070	0.230	5
B12	0.0021	0.001	0.001	0.004	3
C	60 [(b)]	-	-	-	-
D	0	-	0	0	3
E	99.167	19.219	87	121	3
K1	0.005	0.007	0.001	0.010	3
Mineral content (mg/100 g APC on raw basis)	**Average**	**Standard deviation**	**Minimum**	**Maximum**	**No of analyses**
Calcium	3378	435.8	2570	4140	8
Magnesium	148	11.6	130	170	8
Phosphorus	791	54.4	690	840	8
Sodium	10	8.2	0	20	7
Potassium	801	140.8	650	980	7
Zinc	1.97	0.8	1.5	3.6	6
Iron	54.0	8.6	43.5	62.3	4
Copper	0.76	0.1	0.67	0.81	6
Manganese	6.25	0.6	5.4	6.7	6

[(a)] The data of this table are based on results obtained over several years from analyses conducted by several different laboratories: CCPA (2003), CCVE (2007), EVIALIS (2005), France-Lucerne (1980, 1992-1996), IGER (2003), INRA (1980, 1981, 2002), INZO (2003, 2004, 2006, 2007), Ministry of Agriculture, China (2000), UCCAB (1998).

[(b)] Corresponding to ascorbic acid added during manufacturing to prevent oxidation

Table 2. **Results of contaminant analyses for APC obtained from various alfalfa crops**

France lucerne Reference crop	Unit	231 CX 1997	236 CX 1997	PX 199 1996	Eurofins 04-12 2004	Eurofins 04-13 2004	INZO 05-09 2005	INZO 05-10 2005	INZO 06-09 2006	INZO 06-13 2006	INZO 06-14 2006
Pesticide residues											
PCBs	mg/kg	n.t. [a]	n.t.	n.t.	<0.005	<0.005	<0.0015	<0.0014	n.t.	n.t.	n.t.
Coplanar PCBs	mg/kg	n.t.	n.t.	n.t.	<0.005	<0.005	<0.0015	<0.0014	n.t.	n.t.	n.t.
Organochlorinated		n.d. [b]	n.d.	n.d.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Organophosphorus		n.d.	n.d.	n.d.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Total PCBs	mg/kg	-	-	-	-	-	-	-	<0.0017	<0.0006	<0.00027
PAHs (polycyclic aromatic hydrocarbons)											
Total PAHs detected	µg/kg	n.t.	n.t.	n.t.	92.4	124.1	n.t.	n.t.	134	148	327
Total heavy PAHs (>= 5 nuclei)	µg/kg	n.t.	n.t.	n.t.	23	24.2	9.3	9.3	<42	<48	<74
Heavy metals (mg/kg)											
Nickel	mg/kg	<0.0039	<0.0039	<0.0039	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Cadmium	mg/kg	<0.0031	<0.0031	<0.0031	0.18	0.16	0.12	0.243	0.168	0.195	0.13
Lead	mg/kg	<0.0022	<0.0022	<0.0022	1.4	1.3	0.945	0.8	1.937	1.041	1.446
Arsenic	mg/kg	<0.001	<0.001	<0.001	0.6	0.55	< 0.05	0.054	0.308	0.215	0.27
Fluoride	mg/kg	n.t.	n.t.	n.t.	<10	<10	<10	<10	<10	<10	<10
Mercury	mg/kg	<0.005	<0.005	<0.005	0.022	0.017	0.054	0.051	0.03	0.035	0.034
Mycotoxins (µg/kg)											
Aflatoxins B1	µg/kg	<LD =1000	<LD =1000	<LD =1000	n.t.	n.t.	n.t.	n.t.	<LD = 0.05	<LD = 0.05	<LD = 0.05
Aflatoxins B2	µg/kg	<LD =1000	<LD =1000	<LD =1000	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Aflatoxins G1	µg/kg	<LD =1000	<LD =1000	<LD =1000	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Aflatoxins G2	µg/kg	<LD =1000	<LD =1000	<LD =1000	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Vomitoxins	µg/kg	n.t.	n.t.	n.t.	<LD =20	<LD =20	<LD = 20	<LD = 20	n.t.	n.t.	n.t.
Zearalenone	µg/kg	n.t.	n.t.	n.t.	<LD =40	<LD =40	<LD = 3	<LD = 3	< LD = 3	<LD = 3	<LD = 3
Ochratoxins A	µg/kg	n.t.	n.t.	n.t.	n.t.	n.t.	<LD = 0.2	<LD = 0.2	n.t.	n.t.	n.t.
Fumonisin B1	µg/kg	n.t.	n.t.	n.t.	n.t.	n.t.	<LD = 10	<LD = 10	n.t.	n.t.	n.t.
Fumonisin B2	µg/kg	n.t.	n.t.	n.t.	n.t.	n.t.	<LD = 30	<LD = 30	n.t.	n.t.	n.t.
Microbiology											
Mesophile aerobic flora (30°C)	cfu[c] /g	3050	7950	6000	600	35000	100000	92000	16000	n.t.	n.t.
Total coliforms	cfu/g	n.d.	n.d.	n.d.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Salmonella sp	cfu/25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

	g										
Pathogenic staphylococcus	cfu/g	n.d.	n.d.	n.d.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Anaerobic sulfite reducers	cfu/g	n.t.	n.t.	n.t.	n.t.	n.t.	<10	<10	<10	<10	<10
Butyric group clostridium	cfu/g	20	35	75	n.t.	n.t.	93	<3	4	<3	4
Yeasts	cfu/g	n.t.	n.t.	n.t.	<10	<10	<10	<10	60	<10	<10
Mould	cfu/g	n.t.	n.t.	n.t.	60	10	520	10	330	30	10

(a) n.t. = not tested
(b) n.d. = not detected
(c) cfu (colony forming units)

The alfalfa protein concentrate include anti-nutritional factors such as saponins, phytates, and L-canavanine, and secondary metabolites such as phytoestrogens (coumestrol and isoflavones).

The concentration of saponins in the green juice of alfalfa (immediately after the pressing phase) and APC is 2 to 3 % and 0.5 to 1.4 % respectively (Laboratory Nutrinov, tested in December 1999), which is less than contents reported for other leguminous vegetables (3 to 7 %) (Fenwick and Oakenfull, 1983).

Based on the data provided by the applicant, phytates are present in only negligible quantities in APC (< 0.2 g/kg).

According to the applicant, once alfalfa has reached the green stage, the level of L-canavanine is reduced to trace element status. The concentration of non-protein amino acid L-canavanine in APC (3.9 - 4.4 mg/kg, n = 3) according to data provided by the applicant is about double the amount of soy flour (2.1 mg/kg), but much lower than that of other common foods such as lentil flour (2800 mg/kg) or onions (10 000 mg/kg) (Table 3).

Table 3. **L-canavanine concentration in fresh alfalfa seeds, leaves, APC and in comparison with other vegetables consumed by humans (in ppm or mg/kg)**

Alfalfa seeds*	Alfalfa leaves*	APC**	Soy flour**	Lentil flour**	Onions**
80 - 150	10	4.3	2.1	2 800	10 000

* Bruneton, 1999
** Analyses carried out at the Biochemistry laboratory of CHU Reims, 2001

According to the applicant, the production process of APC eliminates more than 80 % of the initial coumestrol. However this cannot be confirmed by data provided by the applicant: a comparison of the test results for the coumestrol content of whole alfalfa with the content in APC which rather indicates that the production process has no impact on the coumestrol content (Table 4).

Table 4. **Coumestrol content of various samples**

Sample	Free coumestrol in ppm (chromatography plate*)	Total coumestrol in ppm (HPLC method**)
Dehydrated whole alfalfa		
August sample 3643	68	100.5
APC		
August sample 3644	82	100
August sample 3646	71	72
July sample 3645	81	100
May sample 3647	31	40
Average of 4 samples (APC)	**66.3**	**78**

* Laboratory: Myco 2B 2007 laboratory method

** Laboratory: Nutrinov 2007 laboratory method

The average total isoflavone content of APC, based on analytical data on 4 samples, was 255 mg/kg, which on average contained 24 mg/kg of daidzein, 101 mg/kg of genistein, and 130 mg/kg of glycitein (Table 5). Based on analytical data provided by the applicant, the total polyphenol content of the extracts is 1 to 2 g per 100 g (Laboratory: Nutrinov, 1999). Thus 10 g/day consumption of APC provides 200 mg of polyphenols per day.

Table 5. **Isoflavones content of whole alfalfa and APC (in mg/kg)**

Isoflavones content mg/kg				
Sample	**daidzein**	**genistein**	**glycitein**	**TOTAL**
Dehydrated whole alfalfa				
August sample 3643	37.9	133.2	150	321.1
APC				
August sample 3644	21.7	29.8	120.6	172.1
August sample 3646	19.6	123.6	139.4	282.6
July sample 3645	19.4	103.4	110.4	233.2
May sample 3647	35.5	148.9	148.8	333.2
APC average	**24.1**	**101.4**	**129.8**	**255.3**

Source : Laboratory: Nutrinov laboratory analyses, March 2007

APC contains xanthophylls such as zeaxanthin, lutein, cryptoxanthin and neoxanthin - about 1,500 mg/kg dry weight with an average distribution of 5, 65, 10 and 10 (%), respectively. Based on the data provided by the applicant (Table 1), a daily dose of 10 g of APC provides 2.2.-7.2 mg β-carotene.

II. Effect of the production process applied to the NF

Currently four factories in France apply this process and produce about 12,000 tons of alfalfa. Alfalfa protein concentrate (APC) is extracted from *Medicago sativa spp. sativa.* Alfalfa is cut, chopped and transported to the factory where it is processed either immediately or max 2 h later in order to avoid enzymatic proteolysis and pigment oxidation.

Afalfa is ground in busters (hammer crushers) or attrition crushers. By passing through an oleaginous-type screw press, alfalfa provides a fibrous residue and press juice (10 % of dry matter). The dry matter of this juice contains about 35 % of crude protein. The press juice (pH 5.8-6.2) is neutralized using alkaline solution up to pH 7.5-8.0. Preheating followed by vapour injection at an average temperature of 85 °C allows coagulation of proteins associated with the carotenoid and chlorophyll pigments. The protein precipitate is separated by centrifugation and thereafter dried at sufficiently low temperatures to preserve the pigments. The thermal treatment allows elimination of bacterial flora and anti-nutritional thermo-sensitive substances such as the antitrypsin factor. Finally the APC is granulated after adding ascorbic acid (600 mg/kg) and stored in inert gas or in cold storage.

The results of contaminant analyses performed on APC obtained from various alfalfa crops are shown in Table 2. APC does not contain any pesticide residues (organochlorine or organophosphorous).

III. History of the organism used as the source of the NF

Alfalfa originates from southwest Asia and was domesticated over 9,000 years ago in the highlands of Caucasus, Iran and Turkey. Alfalfa appeared in France in the 16th century, but was not used extensively until the 18th century, when it was demonstrated that it was a good alternative to fallow and enriched the soil with nitrogen. Today, alfalfa is the most widely grown forage crop in the world.

Dried alfalfa is a raw material rich in proteins and accounts for an average of 10 % of the proteins produced in France and used in animal feed for herbivores, ruminant (bovine, sheep, goat) and other animals (rabbit, horse). According to the applicant, the European production of dehydrated alfalfa is 4.5 million tons. Dehydration of alfalfa was introduced into France in the 1950s and has developed substantially in recent years. In order to increase the nutritive potential of alfalfa in animal feed, an extraction process is carried out prior to dehydration of lucerne. The dry concentrate of alfalfa is high in protein and xanthophyll, and is used in the feed industry for its pigment content. The alfalfa protein concentrate is marketed on the animal feed market under various names including "alfalfa foliar extracts", "alfalfa concentrated extracts", "PX" or "alfalfa green protein". The main market for a production of 12 000 tons/year of alfalfa protein concentrate, is poultry.

IX. Anticipated intake/extent of use of the NF

Alfalfa protein concentrate intended for human consumption has not previously been available on the European market. Outside Europe, alfalfa protein concentrate is authorized in the US, Canada and Mexico. Products are available in various forms (food supplements, drinks, chocolate bars), but mostly in the form of food supplements (capsules, tablets, powder).

The applicant proposes the use of APC as a food supplement with a recommended consumption of 10 g per day. The intended target groups are vegetarians, vegans, the elderly and female adolescents. The applicant proposes that pregnant and lactating women are advised to consume APC under only under medical supervision.

X. Information from previous human exposure to the NF or its source

Alfalfa, the source of the protein concentrate and parts of the plant (leaves, sprouts) have been used for human consumption in the European Community.

A survey was conducted by the applicant in 25 MS in 2006 with feedback from five countries confirming that alfalfa is consumed in the human diet in the form of food supplements and as an ingredient in common foods (soups, salads). However, no quantitative data are available. In USA alfalfa herb and seed are generally recognized as safe (GRAS).

XI. Nutritional information on the NF

Two studies were carried out with laboratory animals (rats, mice) evaluating safety and nutritional benefits of APC. These studies do not meet the international standards required for subchronic or chronic toxicology studies. One study has been carried out in rats in 2006 in order to evaluate whether the alfalfa protein concentrate achieves the same effects (i.e. nutritional benefits) as milk protein. After a period of acclimatisation, during which 12 male Wistar rats were fed pellets containing 30 % protein, the rats were divided randomly into two groups: four rats were given powder containing 13 % milk protein and 8 rats were fed with powder containing 13 % protein from alfalfa protein concentrate for 3 months. The powders had the same energy contents (approximately 15.5 kJ/g) but the contents of lipids and carbohydrates differed considerably (10.58 vs. 5.43 of the energy content and 75.89 vs. 81.44 of the energy content of the powders containing milk protein and APC, respectively). Body composition, hormone profile, metabolic balance, renal function, osseous mineralisation and renal and hepatic histology were measured.

Rats fed on the alfalfa protein concentrate grew slightly faster and reached higher final body weights than rats given milk protein. The excretion of calcium in the urine was increased six times in the group given APC compared to the excretion level in the milk protein group although the major amounts excreted in faeces did not differ much. The calcium intake from APC was lower (264 µg per diet over 24 hours) than from the milk protein (428 µg per diet over 24 hours). The applicant indicated that he had analytical difficulties with the spectrophotometry method applied to measure the calcium content of the APC diet in this rat study and suggested that the real calcium intake in this rat study were higher than indicated (264 µg). As the content of calcium measured in the faeces was similar in the two groups, the applicant concludes that there was a good absorption of calcium from the APC diet. The fact that the bone mineralization from the tibia was identical with the two diets tends to support this conclusion. Considering the reported analytical difficulties the Panel notes that this rat study cannot be considered as a source for a quantitative or comparative conclusion on the calcium balance of APC intake in rats. However it is notes that the APC intake did not lead to a negative calcium balance.

Additional statistically significant differences were found between the two groups, i.e. nitrogen content of the kidneys as well as total cholesterol and insulin levels in plasma. In the opinion of the Panel the nutritional relevance of the observed differences cannot be assessed due to the differences in nutrient contents of the diets administered. The low number of animals per group (4 vs. 8) does not allow reliable conclusions regarding the occurrence of differences between the two groups. The study did not reveal safety concerns.

In a long-term study, 20 female BALC/c mice were fed a diet containing 20 % APC for 7 months. Examination of histological sections of the small intestine and the colon did not reveal any significant modifications. The mice on a 20 % APC diet had a significantly lower body weight than the control mice after seven months of the study (24.5 g vs. 26 g, five mice per group). According to the study report, mice receiving APC also had a reduced feed intake. The authors speculated that the decrease in body weight could be due to the presence of anti-nutritional factors in the alfalfa leaf extract or an artefact. The unattractiveness of the APC diet may also be a factor. The Panel notes, that a lower weight gain was not reported in other studies with mice fed with 20 % APC for 26 weeks (SLE study with 15 BALB/c mice per

group). This SLE study described later was conducted by the same laboratory and operators, with the same mouse strain, at the same time, and with the same feed.

The Panel notes that a higher (statistically not significant) weight gain was reported on rats fed with 13 % protein from APC in a nutritional study. The study did not reveal safety concerns.

Studies of alfalfa protein concentrate in humans have been carried out by the applicant with malnourished or undernourished, deprived, anaemic human beings, mainly children, in some countries (e.g. Peru, India, Congo). These clinical trials focused on the nutritional value of APC. A first clinical study was carried out with 30 children aged 3 to 5 years receiving either 10 g of APC or powdered skimmed milk (with the same protein amount as APC) for one year. A second six month study was carried out with 70 children receiving either 10 g APC or powdered milk. In these studies there were no differences between groups regarding growth. No deleterious effect was observed. A third study compared the effect of 10 g of APC to that of
15 g powdered skimmed milk in 30 children aged 3 to 5. A fourth study compared the effect of 10 g of APC to that of 60 mg tablets of ferrous sulphate for three months in 60 adolescents suffering from anaemia. In these studies the acceptance and tolerance of APC was good.

The alfalfa protein concentrate has been used since 1992 as a food supplement to combat malnutrition in several non-EU countries. 320 tons of APC were consumed in 20 countries throughout the world and no deleterious effect of APC were reported by the various NGOs that used the product.

The Panel considers the animal and human studies focusing on nutritional effects of APC and the history of use as a food supplement without reported adverse effects to be supportive of safe use of the product.

XII. Microbiological information on the NF

The microbial characteristics of APC obtained from various alfalfa crops (Table 2) are in conformity with the European norms applicable to foodstuffs. The contamination of APC by mycotoxins is below the detection limits provided in Table 2.

XIII. Toxicological information on the NF

Whole alfalfa and also APC contain secondary plant metabolites such as phytoestrogens (coumestrol and isoflavones) and anti-nutritional factors such as saponins, phytates, and L-canavanine.

The concentration of saponins in the green juice of alfalfa (immediately after the pressing phase) and APC is 2 to 3 % and 0.5 to 1.4 % respectively (Laboratory Nutrinov, tested in December 1999), which is less than contents reported for other leguminous vegetables (3 to 7 %) (Fenwick and Oakenfull, 1983). Some saponins may slow down growth in young animals by forming a complex with cholesterol. However, according to Malinow et al. (1980) alfalfa saponins act as hypocholesterolemic agents. Considering the low levels of saponins and phytates (< 0.2 g/kg) present in APC compared to other common foods, the Panel has no concerns with respect to saponins and phytates.

Data on the content of xanthophylls in APC are given in Section I. Based on the analytical data provided, a daily dose of 10 g of APC contains about 8 mg of lutein, which is far below the upper range of the acceptable daily intake (ADI) of 0-2 mg/kg bw/day derived by JECFA (2006). The Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) of EFSA previously concluded that lutein, obtained as an extract from marigold flowers (*Tagetes erecta*) and from the natural strains of edible fruits and plants, grass,

lucerne (alfalfa) and *Tagetes erecta* in foods for special medical purposes (FSMP), is not of safety concern under the proposed use levels which are in the range of the regular dietary intake of lutein, provided that it is in compliance with the existing EU specifications of the food additive. The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) in addition noted that data from the USA estimated mean and the 90th percentile consumption of lutein plus Zeaxanthin to 1.7 and 3.0 mg/person/day (NHANES III survey, 1994). For those who met the national recommended daily intake of vegetables an intake of 3.8 (mean) and 7.3 mg/person/day (90 % percentile) was estimated. Higher intakes have been reported from different islands of the South Pacific with a mean lutein intake ranging from 3.9 - 23.6 mg/day among females and from 3.2 - 25.7 mg/day among males (Le Marchand et al., 1995). The Panel concludes that a daily intake of 8 mg lutein from APC supplement is not of safety concern.

ß-carotene

Based on the data provided by the applicant (Table 1), a daily dose of 10 g of APC depending on the alfalfa crop provides from 2.2 mg to 7.2 mg β-carotene. There may be a very small difference between the levels of β-carotene that may confer health benefits (up to 10 mg/d, mainly from natural sources) and those that may produce adverse effects in smokers in the general population (20 mg/day) (SCF 2000). The Panel notes that in March 2009, EFSA has received a mandate from the European Commission to review the existing scientific information on the possible link between the ingestion of β-carotene and cancer enhancement in heavy smokers and to provide an upper safe level for β-carotene for this category of the population (EFSA, 2009a).

L-canavanine

L-canavanine is an L-arginine analogue and can therefore, by replacing this amino acid present in histones influence interactions between nucleic acids and disturb the normal genome function. L-canavanine could interact with an RNA synthesis enzyme.

Based on analytical data provided by the applicant, the concentration of L-canavanine in APC is 4.3 mg/kg dry weight, which amounts to twice that of soy flour (2.1 mg/kg), and is much lower than in other common foods such as lentil flour (2800 mg/kg) or onions (10 000 mg/kg) (Table 3). A concentration of 4.3 mg/kg dry substance would result in a daily intake of 43 microgram at the proposed use level (10 g APC).

In vitro experiments have demonstrated the role of alfalfa L-canavanine in the occurrence of systemic lupus erythematosus (SLE) activation (Alcocer-Varela et al. 1985, Morimoto et al. 1990). The possibility of lupus erythematosus activation following consumption of alfalfa has been examined because of the reactivation of disseminated or systemic lupus erythematosus (SLE) observed in two consumers of alfalfa tablets sold in the USA. These tablets were made with alfalfa seeds and sprouts. L-canavanine which is present mainly in seeds and sprouts of most leguminous plants, has been suspected to be responsible for these lupus cases. The L-canavanine concentration in alfalfa seeds (80-150 mg/kg) or leaves (10 mg/kg,) responsible for the activation of lupus in the observed two cases in USA is higher than in APC (4.3 mg/kg). It should also be noted that the only cases of autoimmune diseases caused by alfalfa are associated with seeds and sprouts, while the novel food ingredient proposed (APC) is made exclusively with the aerial parts of alfalfa.

In order to specify the role played by the alfalfa protein concentrate and alfalfa seed in SLE, a study was carried out on mice. The BALB/c mouse strain selected for the study is one of the two strains most commonly used in lupus studies (Brennan et al. 1998, Satoh et al. 2000). Futhermore, the mice selected were female given that the prevalence of lupus is higher in females than in males. Three groups of 15 mice were included in the protocol and fed 10 g of

APC per day for 26 weeks. A control group was fed on a standard diet containing 14 % milk protein (P14 control diet). One group was fed a diet containing P14 plus 20 % of milled alfalfa seeds and another group was fed a diet containing P14 plus 20 % of the alfalfa protein concentrate. Mouse weight was monitored and serum measurements of various markers were carried out: anti-DNA_{ds} antibodies, titre and incidence of anti-alfalfa serum antibodies, cytokines (TNF-α, INF-α) lupus development, study of populations of Th and Tc lymphocyte cells, B1 and B2 lymphocytes, measurement of dendritic cells in bone-marrow, blood and spleen, and measurement of TLR9 presenting DNA and TLR8 presenting RNA. The anti-DNA_{ds} antibodies appeared only with the diet containing 20 % milled alfalfa seeds. The alfalfa protein concentrate did not cause changes in the SLE markers.

The Panel notes that the L-canavanine content of APC compared to other common foods is low. The Panel concludes that the animal data concerning SLE support the safety of APC regarding activation or reactivation of lupus erythematosus.

Phytoestrogens

Alfalfa is a plant with oestrogen-mimetic activity that has been demonstrated *in vitro* and *in vivo* (Kurtzer and Zu, 1997, Zava et al., 1998). Alfalfa foliar extracts contain phytoestrogens: coumestrol and isoflavones.

The total isoflavone (daidzein, genistein, glycitein) content of APC is 255 mg/kg (Table 5), which is similar to the level of total isoflavonoids present in tofu (250 mg/kg). The suggested consumption of 10 g of APC per day leads to an intake of 2.6 mg of isoflavones which is about 17 % of the average total isoflavone intake via soy-based foods in the French adult population (AFSSA 2005).

Based on the applicant's analytical data, the average coumestrol content of APC is 78 mg/kg, which is comparable to that of the main sources of coumestrol intake (Boker et al., 2002), fresh soy sprouts (71 mg/kg) and split peas (81 mg/kg) (Table 4).

The Panel notes that the coumestrol and isoflavone contents of APC at the recommended daily dose of 10 g of APC, amounting to 0.8 mg and 2.6 mg, respectively are comparable to those of other foods (incl. soy-based foods) widely consumed in Europe. However, the applicant considers that the effects of phytoestrogens on the fertility and the reproductive function are difficult to establish and suggests that pregnant or lactating women and children be advised to avoid consumption of alfalfa protein concentrate.

The Panel concludes that the intake level of phytoestrogens in a 10 g daily dose of APC is lower than from other common food sources and therefore does not raise concern.

The Panel would like to refer to an internal mandate on hazard characterisation of the use of dietary isoflavones and isolated isoflavones from soy or red clover in food supplements (EFSA, 2009b).

***In vivo* safety studies**

The applicant has provided an acute oral toxicity study using male rats. There were no indications of adverse effects after administration of a single dose of 5000 mg APC per kg bodyweight. Information on whether the test material complies with the proposed specification was not given.

No studies on subchronic, chronic, reproduction and developmental toxicity meeting international accepted standards were provided. Thus, information on potential adverse effects after repeated administration of APC from studies using laboratory animals is not available.

The Panel notes that there are no subchronic, chronic, reproduction and developmental toxicological data available on APC. The Panel concludes that other toxicological data concerning levels and effects of anti-nutrients and secondary metabolites (in particular L-canavanine and phytoestrogens) in APC and the information from nutritional effects in animal and human studies are supportive concerning the safety of APC.

Allergenicity

There are no reported cases of allergic reactions to alfalfa in man. However, given the fact that alfalfa is a leguminous plant, cross-reactivity could potentially cause adverse effects in persons who are allergic to foods from the leguminous plant family, peanuts in particular. The applicant has analysed the sequence homology that might exist between alfalfa proteins and known allergens. Out of 526 proteins identified in alfalfa, nine of them presented homology with known leguminous plant allergens.

An *in vivo* study was carried out in order to explore the cross-allergenicity risk with peanut protein. C3H/HeJ mouse strains known to be a good model for studying allergy to peanuts (Li et al. 2000; Bashir et al. 2004; Lifrani et al., 2005) were selected. 24 mice were sensitised to peanuts by intraperitoneal injection of 5 µg of peanut proteins in 100 µL of sterile PBS. IgE peanut specific assays were performed in mice following immunisation. All mice developed IgE antibody responses against peanut. Mice allergic to peanuts were subsequently exposed to APC either orally or intraperitoneally. Oral exposure led to allergy symptoms that are characteristic of stage 1 and 2 allergic reactions i.e. nose and head scratching, skin scratching and reduction in physical activity (Li et al., 2000). Intraperitoneal administration induced mobility difficulties. In conclusion, the ingestion of APC appeared caused slight allergic manifestations in mice sensitised to peanuts.

There are no human data regarding cross-reactivity of APC with peanut using IgE binding or double blind placebo controlled approaches. However, Jensen et al. (2008) reported (relatively weak) skin prick test reactivity to alfalfa in peanut allergic individuals, and histamine release in vitro.

For these reasons, the risk of cross-reactivity in subjects allergic to peanuts cannot be excluded.

DISCUSSION

The main concerns of the Member States were the presence of coumestrol, L-canavanine, and ß-carotene in APC and the potential allergenicity of the product.

Alfalfa foliar extracts contain phytoestrogens (coumestrol and isoflavones) which are known to disturb the reproduction cycle in females. The total isoflavone (daidzein, genistein, glycitein) content of APC is 255 mg/kg, which is similar to the level of total isoflavonoids present soy products. The suggested consumption of 10 g of APC per day leads to an intake of 2.6 mg of isoflavones which is about 17 % of the average total isoflavone intake via soy-based foods in the French adult population (AFSSA 2005). Also the coumestrol content of APC (78 mg/kg) is comparable to that of the main sources of coumestrol intake, fresh soy sprouts and split peas. The Panel notes that for adults and children weighing over 32 kg, the amounts of coumestrol (0.8 mg) and isoflavones (2.6 mg) in the recommended daily dose of 10 g of APC do not exceed the maximum recommended safe levels of isoflavone consumption of 1 mg/kg/day set by AFFSA (2005). The Panel concludes that the intake level of phytoestrogens in a 10 g daily dose of APC is lower than from other common food sources and therefore does not raise concern.

L-canavanine which is present mainly in seeds and sprouts of most leguminous plants, has been suspected of being responsible for systemic lupus erythematosus (SLE) activation. However,

the L-canavanine concentration of APC (4.3 mg/kg) is very low compared to other common food sources such as lentil flour (2800 mg/kg) and onions (10 000 mg/kg). Moreover, alfalfa protein concentrate did not lead to anti-DNA_{ds} antibody production (a SLE marker) in a mouse study designed for lupus. The Panel concludes that the L-canavanine content of APC is of no concern.

A daily dose of 10 g of APC can provide up to 7.2 mg ß-carotene. There may be a very small difference between the levels that may confer health benefits (up to 10 mg/d, mainly from natural sources) and those that may produce adverse effects in smokers in the general population (20 mg/day) (SCF 2000).

An *in vivo* study showed ingestion of APC to cause slight allergic manifestations in mice sensitised to peanuts. Data are lacking on cross-reactivity with peanut using IgE binding, skin prick testing, or double blind placebo controlled FC approaches. The Panel concludes that the risk of cross-reactivity in subjects allergic to peanuts cannot be excluded.

The Panel notes that there are no sub-chronic, chronic, reproduction and developmental toxicological data available on APC. The Panel concludes that other toxicological data concerning levels and effects of anti-nutrients and secondary metabolites (in particular L-canavanine and phytoestrogens) in APC, the information regarding nutritional effects in animal and human studies, and the history of use as a food supplement without reported adverse effects is supportive concerning the safety of APC.

CONCLUSIONS

The Panel concludes that APC as a food supplement is safe for human consumption under the specified conditions of use.

DOCUMENTATION PROVIDED TO EFSA

1. Application under Regulation (EC) N° 258/97 concerning novel foods and novel food ingredients concerning 'Two Leaf extracts from Lucerne' as food (Viridis S.A.).
2. Initial assessment report carried out by France: Initial assessment report relating to a request for an assessment on the use of two leaf extracts of Lucerne as novel food ingredients under EC Regulation 258/97.
3. Member States' comments.
4. Dossier Aliment nouveau: Concentré protéique de luzerne (Réponse aux questions posées en avril 2004 par les autorités européennes)
   Novel Food dossier: Alfalfa protein concentrate (Answers to questions submitted in April 2004 by the European authorities).

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