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Antioxidant Capacity of Cotyledons and Germs of Soybean in Relation to Their Isoflavone Content

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Summary

The aim was to study the relationship between the antioxidant capacity and the isoflavone content of soybean extracts depending on both geographic origin and cultivar. Soybean samples were obtained from two soybean seed parts, germ and cotyledon, from two geographical locations (L1, L2) and two cultivars (Queen, Imari), which gave 8 different samples. HPLC determination confirmed higher isoflavone content in germs than in cotyledons, with higher contents in site L2, and in the Queen cultivar. The antioxidant capacity of soybean samples was determined with two methods, the 2,2-diphenyl-1-picrylhydrazyl scavenging assay and the oxygen radical absorbance capacity assay. The results obtained with both assays showed differences in antioxidant capacity between germ and cotyledon extracts, with a higher antioxidant activity of germ extracts.

Key words: soybean, cultivar, location, isoflavones, antioxidant capacity

Introduction

Soybean has received increasing attention in recent years from health care providers, biomedical researchers and the lay public alike because of its potential role in the prevention of a number of chronic diseases like cancer, coronary heart disease and osteoporosis. Soybean is a rich and relatively unique source of isoflavones, the most common being genistein, daidzein and glycitein, which occur as aglycone, glucoside, acetylglucoside and malonylglucoside (*1*). Antioxidant activities of isoflavones have been described (*2*). Other soybean compounds, like 3-deoxyanthocyanidin, have been studied for their *in vitro* antioxidant capacity (*3*). Fritz *et al.* (*4*) showed the *in vivo* antioxidant activity of dietary soy isoflavones in healthy female subjects as evidenced by significantly lower excretion of urinary lipid oxidation products. It has been mentioned that isoflavones are also present in *in vitro* antifungal activity (*5*), *in vitro* and *in vivo* estrogenic activity (*6,7*), and *in vitro* antimutagenic activity (*8*). Studies measuring the food content and the antioxidant capacity of soybean products and by-products are very important because they generate additional quantitative data to expand existing nutrient databases and they may help in understanding the health benefits. Much attention has been given to the isoflavone analysis of soybean products, notably in commercial soybean foods and in American and Japanese cultivars (*1,9–11*). Recently, Lee *et al.* (*12*) studied the isoflavones of Korean soybean cultivars by examining different genotypes, crop years, locations and storage periods. In the context of the by-products emerging from the soybean food indus-

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try or from the dietary product industry, *i.e.* soybean germs, we were interested in comparing the isoflavone contents of germs and cotyledons regarding their antioxidant capacity. We report here the variation of the isoflavone contents of two cultivars in two locations in France (South East and South West) using HPLC determination with the variation of the antioxidant capacity applying two different methods. The first method uses the efficiency of an antioxidant to scavenge the stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*13*,*14*). The second method, developed by Cao *et al.* (*15*,*16*), then modified by Ou *et al.* (*17*), is called oxygen radical absorbance capacity (ORAC). It measures antioxidant scavenging activity against peroxyl radical induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) at 37 °C using fluorescein as the fluorescent probe.

Materials and Methods

Chemicals

DPPH was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Methanol HPLC grade was obtained from SDS (Peypin, France). Absolute ethanol was from Merck Eurolab Polylabo (Strasbourg, France). Disodium hydrogenophosphate dodecahydrate was from Prolabo. Sodium dihydrogenophosphate monohydrate was from Merck. The fluorescein-free acid, AAPH and (+/–)6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Fluka (Saint Quentin Fallavier, France). Acetone was from Carlo Erba.

Plant material

The two soybean cultivars (Imari and Queen, maturity group I–II) used in this work were field grown in two locations: L1 (Toulouse, France) and L2 (Montpellier, France) in summer 2001. They were sown at the beginning of May and harvested in October, with the same experimental design for the two locations: complete randomized trial with 4 plots for each cultivar. Each plot constituted four lines (4.5 m length; rows spaced by 0.5 m and mean interplant spacing in the rows of 0.15 m). Samples were harvested separately on each plot.

Extraction and sample preparation

Seeds were freeze dried. Germs and cotyledons were separated by hand, milled and extracted with a 10-fold volume of methanol/water (in volume ratio of 80:20) at room temperature in a tumbling mixer for 2 hours. The mixture was centrifuged at 7000 rpm for 10 min (4 °C) and the supernatant filtered through a 0.2-μm membrane filter and 10 μL were injected for HPLC analysis. For the antioxidant activity assays, eight independent extractions were done with 50 mg of each germ or cotyledon powder, using the conditions described above for isoflavone analysis. Then, solvent was evaporated under vacuum and the extracts kept at –20 °C in darkness until tested. Extraction yield was dry matter recovered per 100 g of initial powder.

HPLC determination of total isoflavone content

Reverse-phase analysis (Pump controller P4000, Autosampler AS3000, SpectraSystem, Spectra Physics Analytical Inc., California, USA) was carried out at a flow rate of 1.5 mL/min using a gradient of acidified water (0.1 % trifluoroacetic acid, HPLC grade, SDS, Peypin, France) (solvent A) and acetonitrile (HPLC grade, SDS, Peypin, France) (solvent B). The gradient was as follows: 0–2 min, 100 % A; 4 min, 85 % A; 34 min, 75.5 % A; 41 min, 60 % A; 46 min, 50 % A; 48 min, 0 % A; 50 min 100 % A; 54 min, 100 % A. The UV absorbance (UV1000, SpectraSystem) was monitored at 260 nm and area responses were integrated by PC1000 software (SpectraSystem). Response factors used to quantify the individual isoflavones were determined using external standards (daidzin, genistin, daidzein and genistein from Extrasynthèse, Genay, France; glycitin and glycitein from Plantech, Reading, England; 6'-*O*-malonyldaidzin, 6'-*O*-malonylgenistin, 6''-*O*-malonylglycitin, 6'-*O*-acetyldaidzin, 6'-*O*-acetylgenistin and 6'-*O*-acetylglycitin from Fujicco, Japan).

Antioxidant activity assays

Scavenging activity on DPPH radicals

Mixtures containing soybean extracts and DPPH solutions were incubated in the dark at 37 °C for 30 min (*13*). A solution of 50 mL of DPPH (400 μM) in ethanol was prepared. The eight extracts of soybean germ and cotyledon were dissolved in 5 mL of methanol/water (2:1) mixture. A volume of 1 mL of DPPH in ethanol (400 μM) was added to 500 μL of the dissolved extract in a methanol/water (2:1) mixture and to 500 μL of methanol/water (2:1) mixture. The final volume was 2 mL. The maximum of DPPH absorption in the reaction solvent mixture was at 523 nm. After the incubation period, the absorbance was measured against a blank at 523 nm with a Uvikon 930 spectrophotometer (Kontron Instruments). DPPH solution (2 mL) in a deoxygenated mixture of methanol/water (2:1) served as the blank. The radical scavenging activity of soybean extracts was expressed in terms of IC_{50} (concentration in μM required for a 50 % decrease in absorbance of DPPH radical), calculated at 523 nm. A plot of absorbance *vs.* concentration was made to establish the standard curve and calculate IC_{50} values. All tests were performed in quadruplicate.

ORAC assay

The oxygen radical absorbance capacity assay was carried out as previously reported (*17*). The ORAC assay was adapted to a 96-well plate reader (BGM, Polarstar) with excitation/emission wavelengths at 485 and 520 nm, respectively. The degradation of the fluorescein by the radical formed from AAPH was slowed down in the presence of an antioxidant compound. A phosphate buffer solution (PBS) at pH=7.4 (75 mM) was prepared as solvent and used as a blank. Trolox® was used as standard and a stock solution (250 μM) was prepared by dissolving it in 10 mL of PBS. A working solution of AAPH (153 mM) was prepared in PBS (10 mL). The fluorescein solution (100 nM) was prepared in PBS and was kept in the dark. The 96 well plates were prepared by mixing in each well (final volume 200 μL): 20 μL of the compound tested in 60 μL of PBS and 100 μL of the fluorescein so-

lution. The mixture was incubated at 37 °C for 5 min. Concentrations of compounds were: (*i*) Trolox® 6.25, 12.5, 25 and 50 µM; and (*ii*) soybean extracts 0.01, 0.02, 0.03 and 0.04 g/L. The reaction began with the injection of 20 µL of AAPH solution in PBS in each well and was followed by shaking for 7 s in a 4-mm orbital shaker. Fluorescence intensity was measured for 4 hours. The area under the curve (AUC) was calculated with the formula: $AUC=1+f_1/f_0+f_2/f_0+f_3/f_0+f_4/f_0+...+f_{34}/f_0+f_{35}/f_0$, where f_0 is the fluorescence measured after the first cycle (after shaking) and f_i is the fluorescence measured at time i. The relative ORAC value=[$(AUC_{Sample}–AUC_{Blank})/(AUC_{Trolox}–AUC_{Blank})$]×(concentration of Trolox® µM/g of sample). Sample extracts were analyzed at four concentrations with four concentrations of Trolox®. Then the relative ORAC value was the mean of 4×4 calculations. The procedure was repeated four times to improve the accuracy of the relative ORAC values.

Statistics

The data were analysed as a complete three-factor design with randomised three-way analysis of variance [StatBox software (STAT-ITCF, Institut Technique des Céréales et des Fourrages)] followed by a Newman and Keuls multiple range test to inspect differences at $p<0.05$ or $p<0.01$.

Results and Discussion

Isoflavone extraction yield

It was important to choose extraction conditions which would lead to the best extraction yield for the different seed fractions studied: germ and cotyledon. The extraction solvent was a mixture of methanol/water (80:20, by volume) as reported before (*18*). According to Murphy's study, the methanol/water mixture has a high efficiency for extracting isoflavone compounds from soy germ (*19*). A series of twelve replicated extractions with a mixture of methanol/water (80:20) was performed for each of the eight samples to evaluate the repeatability of the extraction procedure. Extraction yields (*i.e* dry matter recovered after extraction) are expressed as mean percentage±RSD (relative standard deviation) ranging from (19.2±0.3) to (24.0±3.5) % for the cotyledons and from (36.4±3.3) to (38.7±2.4) % for the germs, showing a higher yield for germs (Table 1). A recent study reports a quantitative extraction of isoflavones from soybean with 50 % ethanol at 60 °C using ultrasound-assisted extraction in 20 min (*20*). Nevertheless, this method was not retained in our study, essentially because temperature was known to induce conversion of the malonyl forms to glucosides (*21*). Thus, potential alterations of the antioxidant properties of the samples could be excluded.

Table 1. Extraction yields (initial sample mass=50 mg) and IC_{50} of DPPH$^{\bullet}$ radical absorbance by germ and cotyledon extracts (CI=95 %)

Soybean seed	Extraction yield/%	$\gamma(IC_{50})/(g/L)$
Imari-L1 cotyledon	23.5±3.9	3.60±1.58
Imari-L2 cotyledon	19.2±0.3	2.84±0.14
Queen-L1 cotyledon	24.0±3.5	2.51±0.84
Queen-L2 cotyledon	20.0±0.6	2.54±0.40
Imari-L1 germ	38.7±2.4	1.17±0.23
Imari-L2 germ	37.0±0.8	1.45±0.12
Queen-L1 germ	36.4±3.4	1.21±0.32
Queen-L2 germ	37.0±0.7	1.93±0.26

HPLC analysis

Soy isoflavones have been analyzed by HPLC since the early 1980s. Although isocratic HPLC was attempted initially, the hydrophobicity variation of the isoflavone forms implied that gradient HPLC should be the predominant mode (*22*). Results of isoflavone contents are given in Table 2 as µmol/g of dry matter. The most common soybean isoflavones occur as aglycone, glucoside, acetylglucoside and malonylglucoside derivatives, as shown in Fig. 1. Whatever the seed part, the isoflavones are mainly present in the malonyl form (80 %) or in a glucoside form (20 %). Aglycone and acetyl derivatives represent less than 2 % of the total soybean isoflavones. These values show that the isoflavone content is 6 times higher in germs than in cotyledons. These ranges were already observed in previous works (*9*,*23*). For both locations (L1, L2) and for both seed parts (germ and cotyledon), Imari cultivar had less isoflavone content than Queen (Table 2). The L2 location presents germ and cot-

Table 2. Quantification of different isoflavones and isoflavone total content of germ and cotyledon extracts (in µmol/ g of dry matter) with HPLC analysis (means from 4 field plots and standard deviations)

Soybean meal extracts	Glucosyl			Malonyl		
	Daid	Glyc	Ginist	Daid	Glyc	Ginist
Imari-L1 cotyledon	0.17±0.01	0.01±0.00	0.39±0.01	1.11±0.06	0.05±0.01	2.83±0.06
Imari-L2 cotyledon	0.06±0.00	0.01±0.00	0.12±0.01	0.30±0.04	0.03±0.00	0.76±0.07
Queen-L1 cotyledon	0.77±0.04	0.01±0.00	0.70±0.03	3.67±0.22	0.77±0.01	3.46±0.14
Queen-L2 cotyledon	0.26±0.03	0.01±0.00	0.30±0.00	0.30±0.15	0.05±0.00	1.45±0.11
Imari-L1 germ	1.66±0.15	1.66±0.12	0.37±0.07	13.89±0.86	6.67±0.38	3.57±0.23
Imari-L2 germ	1.99±0.22	1.49±0.09	0.70±0.13	8.89±0.40	4.54±0.12	2.40±0.17
Queen-L1 germ	1.28±0.05	4.20±0.11	0.72±0.13	9.95±0.40	12.46±0.27	3.64±0.11
Queen-L2 germ	1.50±0.08	4.23±0.09	0.59±0.01	6.78±0.32	10.30±0.16	2.84±0.05

Table 2. continued

Soybean meal extracts	Acetyl			Aglycon			Total isoflavones
	Daid	Glyc	Ginist	Daid	Glyc	Ginist	μmol/g
Imari-L1 cotyledon	0.02±0.01	0.00±0.00	0.01±0.01	0.01±0.00	0.00±0.00	0.02±0.00	4.63±0.01
Imari-L2 cotyledon	0.02±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.00	0.01±0.00	1.32±0.01
Queen-L1 cotyledon	0.04±0.00	0.00±0.00	0.00±0.00	0.03±0.00	0.00±0.00	0.02±0.00	9.47±0.04
Queen-L2 cotyledon	0.02±0.00	0.00±0.00	0.00±0.00	0.05±0.00	0.00±0.00	0.04±0.00	2.48±0.02
Imari-L1 germ	0.08±0.00	0.08±0.01	0.08±0.01	0.10±0.01	0.00±0.00	0.03±0.00	28.19±0.15
Imari-L2 germ	0.05±0.01	0.09±0.01	0.21±0.01	0.15±0.04	0.00±0.00	0.04±0.00	20.55±0.10
Queen-L1 germ	0.06±0.01	0.14±0.01	0.11±0.01	0.08±0.01	0.00±0.00	0.04±0.00	32.68±0.09
Queen-L2 germ	0.05±0.05	0.25±0.01	0.27±0.02	0.18±0.00	0.00±0.00	0.06±0.00	27.05±0.07

Daid: daidzein, Glyc: glycitin, Ginist: ginistein

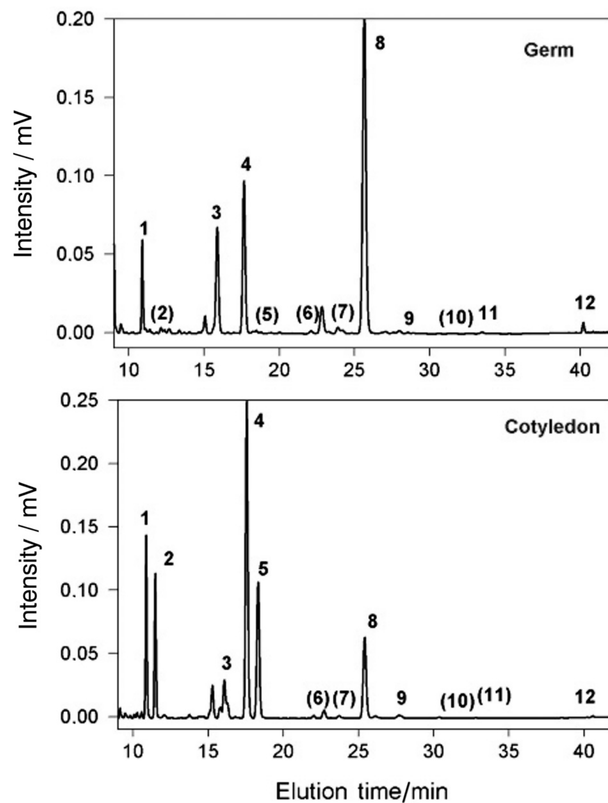

Fig. 1. HPLC analysis of isoflavones in soy fractions. Absorbance was measured at 260 nm. Peak identification: 1 daidzin, 2 glycitin, 3 genistin, 4 malonyl daidzin, 5 malonyl glycitin, 6 acetyl daidzin, 7 acetyl glycitin, 8 malonyl genistin, 9 daidzein, 10 glycitein, 11 acetyl genistin, 12 genistein. Parentheses indicate that this isoflavone was not detected

yledon isoflavone with lower contents for Imari and Queen genotypes than the L1 location. Then, it appears that cotyledon and germ levels depend on two variation factors: the cultivar and the part of the seed. The combination of these parameters can influence the cotyledon isoflavone level by a factor of six and the germ isoflavone level by a factor of 1.5. These different isoflavone levels from 2.7 to 65.7 µmol/g are often mentioned in published studies (*10–12,23–28*). Genotype effects could explain at least 50 % of this variability. Concerning the environmental effects, the above authors accordingly considered that temperature induces a decrease of the isoflavone levels, and that watering, mainly late watering (thunderstorms, rain, *etc.*) could increase isoflavone contents (*29*). Location L2 is situated in the southeast of France where temperatures are hot during autumn with long dry periods (*30,31*). Location L1 is, on the contrary, in the southwest of France where rainfall is higher between summer and autumn.

DPPH assay

The DPPH assay has been widely used to test the free radical scavenging ability of various natural products (*32,33*) because it is the simplest method that measures the ability of antioxidants to intercept free radicals. Both percentage of inhibition (I/%) and inhibition concentration IC_{50} of DPPH absorbance by soybean extracts were determined at 523 nm. According to Table 1, the antioxidant capacity of germ extracts was greater than those of cotyledon extracts. The percentage of inhibition decreased in the following order: Imari-L1>Imari-L2~ Queen-L1>Queen-L2. Imari-L1 germ presented higher antioxidant capacity than Queen-L1 germ. Smaller IC_{50} values for germ extract mean that germ extracts present greater antioxidant capacity than cotyledon extracts, as found with the percentage of inhibition values. For germ extracts, the IC_{50} values decreased in the following order: Queen-L2>Imari-L2>Queen-L1>Imari-L1. Strong interactions with α=1 % were found between locations (L1×L2), and between cultivars (Imari×Queen) for germ extracts. The strongest effect was then observed for Imari-L1 germ extract with a value of (1.17±0.23) g/L, but no interaction between location and cultivar (L1×Imari) for germ extracts was determined statistically. For cotyledon extracts, the IC_{50} values decreased in the following order: Imari-L1>Imari-L2>Queen-L1~Queen-L2. No significant difference was observed between locations (L1×L2) or between cultivars (Imari×Queen) for cotyledon samples according to their antioxidant pool results. With DPPH IC_{50} results, a significant difference (α=1 %) was found among the soybean seed parts, germ and cotyledon, confirming that germ extracts have a better antioxidant pool than cotyledon extracts. The statistical study shows two strong interactions between locations (L1×L2) and between cultivars (Imari×Queen) only with germ

extracts. DPPH results confirm that the antioxidant pool of germ extract was higher than that of cotyledons and show that among germ extracts, location L1 presented a better antioxidant pool than location L2.

ORAC assay

Unlike other popular antioxidant activity methods, the improved ORAC assay with fluorescein as fluorescent probe provides a direct measurement of hydrophilic chain-breaking capacity against peroxyl radical. The same soybean germ and cotyledon extracts as those used in the DPPH assay were employed for the ORAC assay. As shown in Fig. 2, soybean germ extracts slowed down fluorescence decay more than cotyledon extracts at the same concentration, indicating that the germ had a better antioxidant capacity than cotyledon. Fig. 3 shows the linearity of the AUC with a concentration range from 0 to 0.05 g/L for both Imari-L1 germ and Imari-L1 cotyledon extracts. Fig. 4 compares and underlines the higher antioxidant capacity of germs over cotyledons. The values obtained by ORAC assay were statistically studied using the average values of the three parameters considered: seed part, location and cultivar. The Newman--Keuls test showed two highly significant differences with α=1 %, (*i*) between seed parts (germs and cotyledons), whatever the location or the cultivar, and (*ii*) between locations (L1 and L2), whatever the considered seed part or the cultivar. Statistical test showed a significant difference with α=5 % only for the cultivar factor. Germ extracts presented the highest ORAC values. Isoflavones are typical molecules isolated from leguminous plants. They are postulated to play important roles both as defensive compounds against pathogenic microorganisms and as chemical signals in symbiotic nitrogen fixation (*34,35*). Moreover, during the germination, a transient increase of isoflavone content was observed during the early germination (*36*). Thus, germs accumulated more isoflavones than the cotyledons, probably to get advantage in symbiosis activity and to protect themselves from soil pathogens.

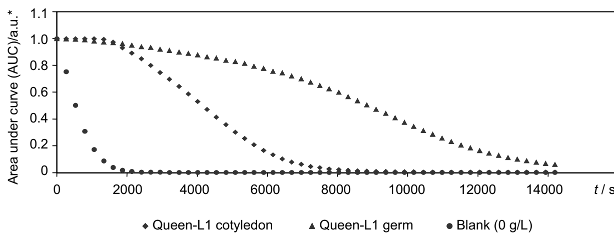

Fig. 2. Effects of germ and cotyledon soybean extracts (0.02 g/L) on fluorescein fluorescence decay curve
*a.u. arbitrary units

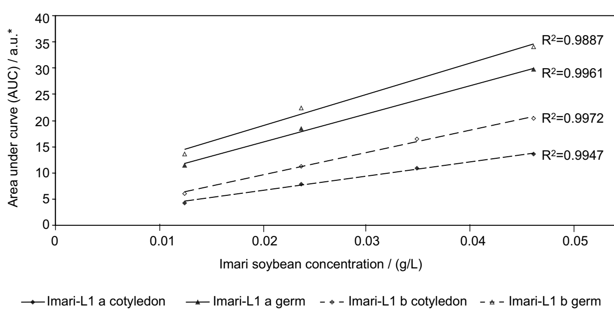

Fig. 3. Effects of the Imari extract concentration on linearity of the area under fluorescence decay curve (a: extract 1 and b: extract 2)
*a.u. arbitrary units

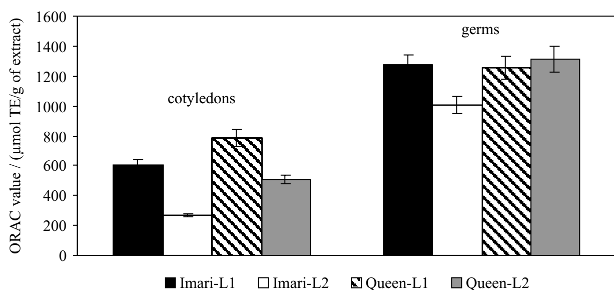

Fig. 4. ORAC values of germ and cotyledon soybean extracts

According to Fig. 4, cotyledon extracts from Queen cultivar from the location L1 had a greater antioxidant capacity than cotyledons from Imari cultivar from the location L2. We can conclude that the antioxidant capacity of cotyledon extracts depended on location and on cultivar. But, the statistical study did not reveal any interaction between the soybean part (germ or cotyledon) and/or the location and/or cultivar. So, the ORAC assay did not allow us to find a direct effect of either location or cultivar on the antioxidant capacity of cotyledon extracts.

Conclusion

The results of this study on the variation of soybean isoflavone levels suggest that it may be feasible to improve soybean cultivars to reach higher levels of antioxidant substances. The measurement of the antioxidant capacity of soybean germs and cotyledons is important to quantify the intake of natural antioxidants in a dietary supplement. Additionally, these studies help in the selection of cultivars and in the study of the health relevance of soybean products or by-products in the field of soy food industry.

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