Composition variability in soy-derived dietary supplements designated for menopausal symptom prevention

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Abstract: *An increasing number of soy isoflavone supplements are commercialized and many investigations are conducted to define their real impact on human health. The presence of other compounds (soyasaponins, phytosterols, polyunsaturated fatty acids...) is rarely considered when discussing the bioactivity of an isoflavone-enriched product. Moreover, the process used can modify the phytochemical content and composition of the final product. This report evaluated the variability in content and composition of isoflavones as well as soyasaponins, proteins, fatty acids and α-galactooligosaccharides of 25 soy based dietary supplements. For isoflavones and soyasaponins, analyzed by high performance liquid chromatography coupled with an ultraviolet detector (HPLC/UV), the 'intra product' variability was investigated by analyzing two different lots of five of these 25 dietary supplements. Proteins were determined through the quantification of total nitrogen by an elementar analyzer, fatty acids by gaz chromatography, and α-galactooligosaccharides were analyzed using a refractive index detector.*

These components showed a high variability: the total isoflavone contents, expressed as aglycone equivalents, varied from 4.4 mg/g (16.7 μmol/g) to 95.3 mg/g (365.6 μmol/g), and the isoflavones/soyasaponins ratio varied from 0.9 (more saponins than isoflavones) to 12.9. In the same way, the protein contents ranged from 0.4 to 42.9%, and the lipid contents from 1.6 to 20%. A high variability was also observed in the profiles of these metabolites. All these differences allowed us to distinguish two main classes of dietary supplements; the whole seed based products, with genistein occurring as the major isoflavone, and the soy germ based products, with a low genistein but high glycitein content. Soy germ and whole seed based products displayed also very contrasted profiles for the other components. An additional variability, more related to the process used, was detected when the conjugation profiles were taken into account, as both heat and pH can selectively affect isoflavone and soyasaponin conjugation. Analyzing different lots revealed the importance of raw material and process cumulated variabilities: total isoflavones displayed large decreases (30%) or increases (17%), and soyasaponin contents varied from 80% decrease to 30% increase. The characterization of the complex phytochemical mixture of a natural dietary supplement needs to be clarified since some active compounds may be responsible for additive, synergistic or antagonist effects only attributed to isoflavones. At least, given its influence on the end product composition, the information about the raw material origin (germ or whole seed) is of great interest.

Key words: *isoflavones, dietary supplement, soy germ, soyasaponins*

Introduction

Several epidemiological and clinical studies associate significant health benefits with soy-derived product consumption. Benefits such as prevention of menopausal symptoms [1, 2], including attenuation of bone loss during the menopausal transition [3], colon and breast cancer [4-8] and cardiovascular diseases prevention [9] have been particularly attributed to the presence of isoflavones.

Isoflavones found in soybean and soy derived products have three distinct structural forms derived from the aglycone molecules genistein, daidzein, and glycitein, and specific structure-related biological activity [10, 11]. The three aglycones may be derivatized to corresponding 7-O-β-glucosides (genistin, daidzin and glycitin), and subsequently malonylated (6''-O-malonylgenistin, 6''-O-malonyldaidzin and 6''-O-malonylglycitin) *(figure 1)*. The acetyl forms arise from malonyl conjugates mainly under heat treatments [12-14]. Consequently, each aglycone is represented by four conjugated forms, giving the twelve isoflavones found in soy based products. The variability of total isoflavones in soy foods or dietary supplements has been reported in several studies [15-17], but information concerning the variability of isoflavone profile in these products remains unclear. Most of the reviews and meta-analysis evaluating the effect of isoflavone treatments in menopausal symptoms have not taken into account neither the chemical identity of individual isoflavone molecules concentrated into the tested products nor the compositional ratio of isoflavones, nor their ability to be metabolized into a more efficient molecule such as equol [18, 19]. However, since

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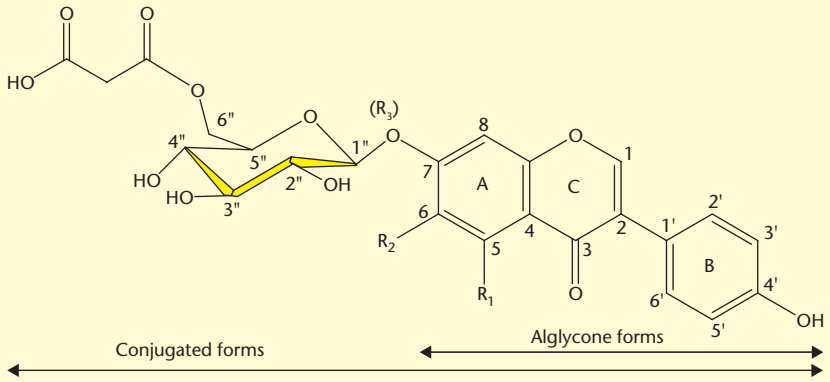


	R_1	R_2	R_3
Daidzein	H	H	OH
Daidzin	H	H	O-gluc
Malonyl Daidzin	H	H	O-gluc-6"-O-malonyl
Acetyl Daidzin	H	H	O-gluc-6"-O-COOH
Genistein	OH	H	OH
Genistin	OH	H	O-gluc
Malonyl Genistin	OH	H	O-gluc 6"-O-malonyl
Acetyl Genistin	OH	H	O-gluc 6"-O-COOH
Glycitein	H	OCH3	OH
Glycitin	H	OCH3	O-gluc
Malonyl Glycitin	H	OCH3	O-gluc 6"-O-malonyl
Acetyl Glycitin	H	OCH3	O-gluc 6"-O-COOH

Figure 1. *Structure of soybean isoflavones.*
The three aglycones forms (daidzein, or 4',7-dihydroxyisoflavone ; genistein, or 4',5,7-trihydroxyisoflavone; and glycitein, or 4',7-dihydroxy - 6-methoxyisoflavone) can be glycosylated, giving the 7-O-β-glycoside forms, and subsequently malonylated, giving the labile 6"-O-malonyl- β -glucosides. The acetyl forms are not genuine forms: they are produced after heat induced decarboxylation of the malonyl.

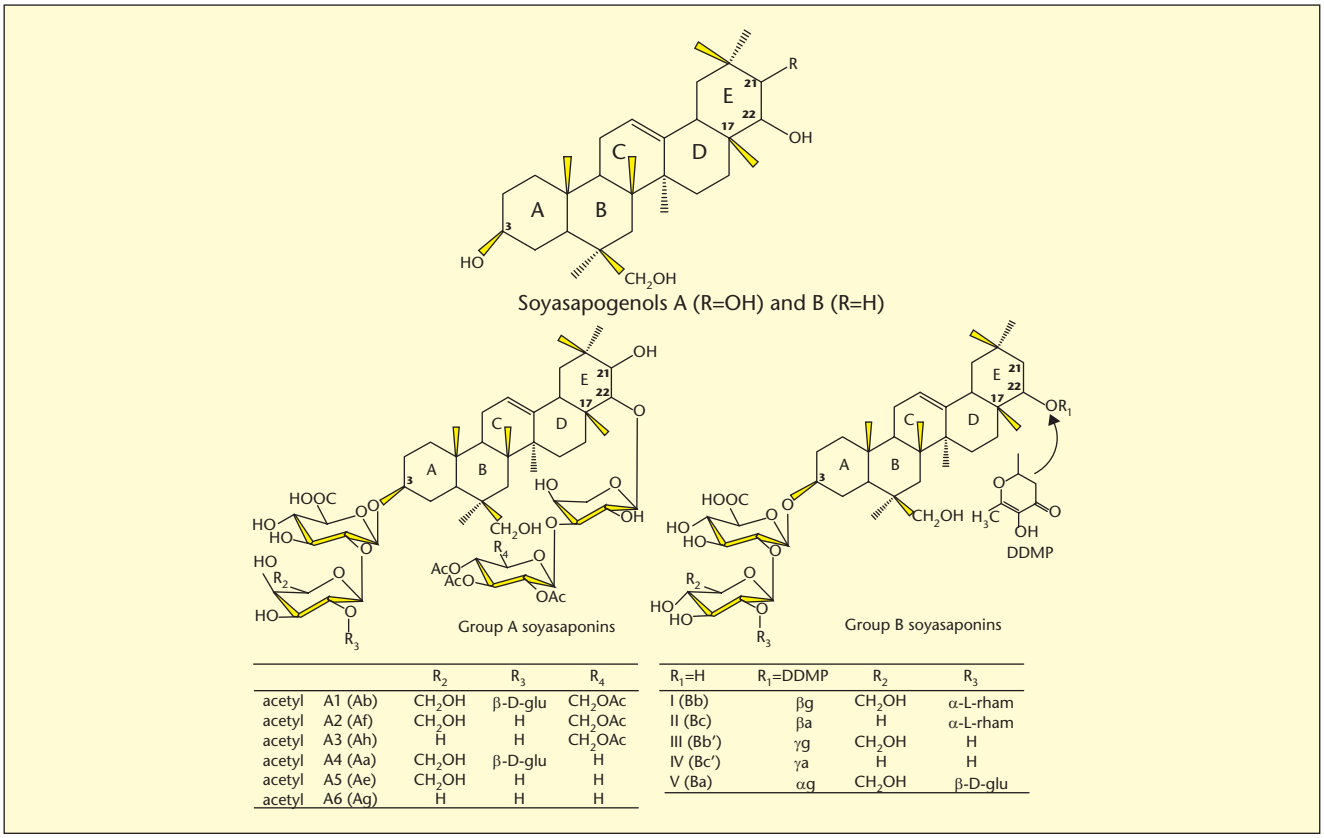
only aglycones are absorbed, the conjugated forms are hydrolyzed in human gut, leading to a delayed bioavailability of the glycosides. Consequently isoflavone pharmacokinetics, may strongly depend on the isoflavone composition of the soy based product absorbed [20, 21]. There are scarcely studies taking into account the presence of other components in isoflavone dietary supplements, despite their possible effects on human health even at low concentrations. Indeed, these compounds may confer additive, different or unsuitable biological properties to the isoflavone dietary supplements. For instance, decreased serum lipid contents may be dependent on interaction between isoflavones, saponins and proteins [22, 23].

Soyasaponins are almost as abundant as isoflavones in the soybean seed. They contain an aglycone (soyasapogenol A or B), linked to one (monodesmosidic) or two (bidesmosidic) oligosaccharidic chains *(figure 2)*. Soyasaponins A are mainly expressed in the germ. They are supposed to be responsible for undesirable bitter and astringent taste in soy-food products, due to the presence of acetyl groups on the last sugar of their specific oligosaccharidic chain [24, 25]. Group B monodesmosidic soyasaponins might be responsible for the health contributing activities of soyasaponins [26, 27]. The genuine soyasaponins B (*i.e.* αg, βg, βa, γg and γa), are conjugated with a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)-group, and predominate their corresponding non-DDMP structures (*i.e.* V, I, II, III and IV respectively), formed as artifacts during heat or alkaline treatments [28]. Soyasaponin V and its corresponding DDMP form, αg, are found exclusively in the germ, while soyasaponins II and βa are mainly located in the cotyledons [27, 29]. Soyasaponins I and βg are the major soyasaponins in both parts of the seed. The ratio of soyasaponins in soygerm cotyledon is around 9 : 1 [30]. The effects of soyasaponins on human health have received much less attention than isoflavones, and little is known about their bioavailability. This is mostly due to the lack of purified standards of the different conjugated forms of soyasaponins, and consequently to the awkward quantification of these forms. *In vitro* and *in vivo* studies have shown that soyasaponins exert hypocholesterolemic [31], hepatoprotective [32], or anticarcinogenic [33] activities, suggesting that these phytochemicals may play a role in preventing human diseases.

It is also known that dietary polyunsaturated fatty acids (PUFAs) have an effect on plasma lipid levels, cardiovascular and immune function, or neuronal development [34]. A recent study comparing the effect of an isoflavone extract alone or combined with a PUFAs supplement on menopausal symptoms, has shown a greater efficacy of the isoflavone plus PUFAs treatment, probably due to the ability of Omega-3 fatty acids to reduce the number of hot flushes through an action on neuronal membranes [35]. The fatty acids profile of the whole seed presents high oleic (24%) and linoleic acid (54%), and low palmitic (11%) and linolenic acid (7%) contents [36], whereas in the germ, linolenic and linoleic acids represent about 17% and 53% of total fatty acids, respectively [37].

Soy peptides can also have hypocholesterolemic [38, 39], or anticarcinogenic activity [40]. Thus, variability of the dietary supplements in protein content or composition can also affect their biological activity.

The health effect of soybean compounds is closely related to the nature of their digested products. For example, only a third of the human population is able to metabolize the isoflavone daidzein into equol, which has a greater phytoestrogenic and antioxidant activity than daidzein. This conversion is closely related to the nature of gastrointestinal microflora, which may in turn be influenced by dietary habits. It has been shown on an ovariectomized mice model that the combination of dietary fructooligosaccharides (FOS) with isoflavones prevents osteoporosis [41]. This suggests that particular components with proven prebiotic activity can influence the bioavailability of isoflavones, and consequently there bio-



		R_2	R_3	R_4
acetyl	A1 (Ab)	CH_2OH	β-D-glu	CH_2OAc
acetyl	A2 (Af)	CH_2OH	H	CH_2OAc
acetyl	A3 (Ah)	H	H	CH_2OAc
acetyl	A4 (Aa)	CH_2OH	β-D-glu	H
acetyl	A5 (Ae)	CH_2OH	H	H
acetyl	A6 (Ag)	H	H	H

R_1=H	R_1=DDMP	R_2	R_3
I (Bb)	βg	CH_2OH	α-L-rham
II (Bc)	βa	H	α-L-rham
III (Bb')	γg	CH_2OH	H
IV (Bc')	γa	H	H
V (Ba)	αg	CH_2OH	β-D-glu

Figure 2. *Structure of soyasapogenol A, B and of their corresponding glycosylated forms.*
Group A soyasaponins are mainly found in soy germ. Their acetyl conjugations are thought to be responsible for the bitter or astringent taste of soyfoods. In the group B soyasaponins, the loss of the DDMP group is induced by heat or alkaline treatments. The non-DDMP forms are probably not genuine soyasaponins.

logical effects. In the soybean seed, raffinose and stacchyose are the most abundant α-galactooligosaccharides, and have been shown to exert an effect on the metabolic activity of the intestinal microflora [42].
Dietary supplements are produced from a great variety of soy raw materials, including meal extracts, whey concentrates, or germ extracts presenting variability in content and composition of potentially active compounds. Moreover, changes in the industrial process can modify the composition of the end product, underlying the need of precise information concerning the levels and nature of potentially active compounds in the dietary supplements in order to determine health claims.
The aim of this work was to (i) determine the variability in content and composition of potentially active components, such as saponins, proteins, fatty acids and α-galactooligosaccharides in 25 isoflavone dietary supplements (ii) evaluate the influence of process changes concerning 5 of these products.

Materials and methods

Soy based dietary supplements and chemicals

Twenty five soy isoflavone dietary supplements were purchased locally. For five out of them (P6, P7, P8, P9, P14), a sample coming from a different batch was purchased after a delay of 6 to 9 month. Before analysis, the products were stored in dry conditions, at 4°C. HPLC-grade solvents and other chemical reagents with proper purity were used (SDS, Peypin, France). Purified standards of daidzin, genistin, glycitin, daidzein, genistein, glycitein, soyasapogenol A and soyasapogenol B were provided by Chromadex (Santa Ana, CA, USA). Purified standard of arachidic acid, glucose, raffinose and stachyose were provided by Sigma Aldrich (Sigma, Steinheim, Germany).

High performance liquid chromatography (HPLC) analysis of isoflavones

Duplicate samples of ten tablets or capsules were ground. An aliquot (0.1 g) was extracted with 80% aqueous methanol (8 mL) for 2 h at room temperature. The residue was removed after centrifugation and decantation of the clear supernatant. The final solutions were filtered (0.2 μm, Acrodisc Syringe Filters, GHP membranes) and analyzed by HPLC with a P4000 pump controller, AS3000 autosampler and UV2000 detector (Spectra Physics Analytical Inc., Fremont, California, US). The analytical column, 250 × 4.6 mm i.d., 5 μm, Satisfaction RP-C_{18}-AB (Cluzeau, Sainte Foy La Grande, France) was kept at 30 °C. The mobile phases were 0.05% (v/v) trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as reported by Murphy *et al.* [43] with minor modifications: solvent B increased from 0 to 15% in 2 min, then to 18% in 4 min, to 24.5% in 26 min, to 40% over 7 min, then to 50% in 1 min, and finally increased to 100% in 6 min. The gradient program recycled back to the initial state of 100% solvent A in 2 min. UV absorbance was monitored at 260 nm. The injection volume was 10 μL and the flow rate was 1.5 mL/min. Calibration curves were established using the β-glucosides and aglycone isoflavones standard molecules. Due to their unstable structures, the malonyl- and acetyl- conjugated isoflavones were not used as external standards. Their response factor was calculated from those of their corresponding β-glucoside forms, correcting them in a molecular mass ratio. Since the

malonyl- and acetyl- group do not contain an ultraviolet chromophore, we hypothesized that the absorption properties of the β-glucoside structures at 260 nm should not be modified by a malonyl- or an acetyl-conjugation, and that their response factor was only depending upon their molecular weight [44]. Isoflavone were expressed in their massic concentration as aglycone equivalents, as well as in μmol/g of Dry Weight, according to the recommendations made after the Fifth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease [19]. The extraction recovery of isoflavones was determined by repeating extractions from the residue left from pre-extractions, until the total isoflavone concentration remained constant. With a dilution coefficient of 4% (w/v), the extraction recovery of isoflavones was higher than 95% when extracted only one time.

HPLC analysis of soyasapogenols A and B

The aqueous-alcoholic extracts prepared for isoflavone determination were also used for the quantification of soyasapogenols A and B, as described in a previous study [45]. Briefly, each filtered extract (4 mL) was transferred into a hemolysis tube. A methanolic solution of 1 N HCl (5 mL) was added, and the mixture was submitted to acid hydrolysis at 85 °C for 6.5 h in a water bath to release the aglycone soyasapogenols from the soyasaponins. The extraction and hydrolysis steps were duplicated. After cooling at room temperature, the solutions were analyzed by reverse-phase HPLC with the same column and apparatus as used for isoflavone analysis. Solvent A consisted in acetonitrile: 1-propanol: water: acetic acid (80:6:13:0.1) and solvent B was 100% acetonitrile. Solvent A was pumped isocratically for 15 min. Then solvent B increased to 100% in 2 min and remained at 100% for 2 min. The gradient recycled back to the initial state of 100% solvent A in 3 min. The injection volume was 50 μL and the flow rate 0.9 mL/min. UV absorbance was monitored at 205 nm. An extern calibration was realized using the two purified standards of soyasapogenols A and B. To validate the analytical procedure, the extraction recovery was determined using betulin as internal standard, and multiple hydrolysis times were tested to determine the hydrolysis efficiency. The extraction recovery was higher than 90% after the acid hydrolysis step. The best compromise to produce the optimal quantity of sapogenols in our dilution conditions without inducing their degradation was to stop the reaction after 6.5 h of hydrolysis at 85 °C [45]. Prior to this time, the formation of the 2 sapogenols was not quantitative, whereas a longer hydrolysis promoted their alteration. Ten replications of the entire analytical procedure, carried out successively on the whole soybean seed and on the germ, led to a within-day variability of total soyasapogenols lower than 7.3% in the whole seed and 4.7% in the germ, which indicates a good repeatability of the sample preparation. The between-day variability was found lower than 10.9% by repeating the within-day assay 3 times over a month. These results were detailed in a previous study [45].

Quantification of total proteins

The total protein percentage in the different dietary supplements was determined according to the method of DUMAS [46] through the quantification of total nitrogen using an elementary analyzer (NA 2000 Nitrogen analyzer, Fisons Instruments, Milano, Italy). This method involves a total combustion of the matrix under oxygen, followed by a reduction of nitrogen oxides in N_2 and trapping sulphur components and excess oxygen by copper. The carrier gas was helium. The oxidation tube, containing Chromium oxide, cobalt oxide and quartz wool, was heated to 900 °C, while the reduction tube, containing copper and held back by the quartz wool, was heated to 750 °C. The total nitrogen level was determined using a catharometer detection (NA 2000, Nitrogen analyser, Fisons Instruments, Milano, Italy), which was kept in a thermal chamber at 60 °C. Pure methionine was used as standard, and the coefficient converting nitrogen content into total soy-derived proteins was 6.25. This method is validated on dairy products by the norm ISO 14891 (2002).

Gas chromatography analysis of fatty acids

The total lipidic fraction of each sample (10 g) was extracted in a soxhlet apparatus by a mixture of *n*-hexane/ chloroform (70: 30 v: v) under refluxing for 4 h. The solvents were then evaporated under vacuum at 45 °C. The extracted oils were stored at – 20 °C prior assay. Approximatively 0.1 g crude oil, and 1 mL of arachidic acid (1 mg/mL) used as internal standard were diluted in 5 mL NaOH (0.5 M in methanol) and submitted to saponification for 30 min at 65 °C. 2.5 mL of boron trifluoride (12% in MeOH) were added, and the methylation was achieved in a water bath for 3 min at 65 °C. After cooling, 5 mL of distilled water were added, followed by 10 mL *n*-hexane. After decantation, the organic phase containing the fatty acids was analyzed by capillary gas chromatography (GC 8000 series, Fisons, Milano, Italy) using a flame ionization detector (FID) at P(H_2) 100 kPa and P(air) 60 kPa. Samples (1 μL) were introduced by direct injection into a 30 m × 0.25 mm i.d × 0.25 μm film thickness J&W Scientific capillary GC column (Cluzeau, Sainte Foy la Grande, France). The initial oven temperature of 185 °C increased at 5 °C/min to 200 °C where it was held for 20 min. Inlet and flame-ionization detector temperatures were 200 °C. Palimitic (16: 0), stearic (18: 0), oleic (18: 1), linoleic (18: 2) and linolenic (18: 3) acids were identified using their corresponding purified standards, and quantified through an internal calibration using arachidic acid (20: 0).

HPLC analysis of α-galactooligosaccharides

Stachyose, verbascose and raffinose are the only α-galactooligosaccharides found in soybean. An aliquot of each soy isoflavone dietary supplements (0.1 g) was diluted in distilled water (5 mL) and stirred for 1 hour at room temperature. The residue was removed after centrifugation and decantation of the clear supernatant. Extraction was duplicated for each sample. The final solutions were filtered (0.45 μm) and analyzed by HPLC (ICS-Bischoff, Toulouse, France) using a HDO C18 analytical column (250 × 4.6 mm i.d, 5 μm, Interchim, Montluçon, France). The mobile phase was distilled water, pomped isocratically at a flow rate of 0.5 mL/min for 21 min. The analytical column was finally washed with a solution of 20% MeOH for 5 min. The injection volume was 10 μL. The α-galactooligosaccharide contents were determined by a refractive index detector (ICS-Bischoff, Toulouse, France). A calibration curve was established using β-D-glucose and used for the quantification of the α-galactooligosaccharides stachyose, verbascose and raffinose.

Results

Isoflavone content and profile

Soy isoflavones are derived from 3 distinct aglycone molecules, daidzein, genistein and glycitein, thus, for each of them, the molar contents (μmol/g) of the conjugated forms were pooled and expressed as percentages of the total isoflavone concentrations. Total isoflavone concentrations, aglycone and conjugation profiles displayed great variability between the tested products *(table 1)*. The total isoflavone concentrations ranged from 16.7 μmol/g (P1) to 365.6 μmol/g (P12). The specific isoflavone profiles obtained for each product reflected the nature of the raw material used for processing. Indeed, a major distinction has been established between the whole seed *versus* germ based raw materials. In the germ, daidzein and glycitein conjugates represent 40-60% and 25-40%, respectively, of the total isoflavones, whereas in the cotyledons, genistein conjugates are

Table 1. *Isoflavone content and profile in 25 soy based dietary supplements.*

			Isoflavone profile				Conjugation profile		
		Total en μmol/g (mg/g aglycone equivalent)	**%daidzein**	**%genistein**	**%glycitein**	**%aglycones**	**%β-glycosides**	**%malonyl glycosides**	**%acetyl glycosides**
Soy germ based products	P1	16.7 (4.4)	59.0	12.8	28.2	3.7	52.2	3.5	40.7
	P19	37.3 (9.8)	65.1	10.6	24.3	4.6	50.1	3.9	41.3
	P21	40.5 (10.81)	50.9	14.8	34.3	6.7	56.0	4.4	32.9
	P25	60.6 (16.2)	50.8	14.0	35.1	3.0	93.2	1.1	2.7
	P24	63.6 (16.8)	59.0	14.1	26.9	4.7	58.2	2.2	34.9
	P23	67.6 (17.9)	63.0	9.3	27.7	5.8	55.2	7.8	31.2
	P14	**117.4 (31.2)**	**54.4**	**16.1**	**29.5**	**6.6**	**81.8**	**1.5**	**10.2**
	P14'	*101.0 (26.9)*	*54.1*	*15.6*	*30.3*	*3.0*	*82.9*	*1.8*	*12.4*
	P8	**149.8 (39.7)**	**57.8**	**14.5**	**27.7**	**4.4**	**63.3**	**30.3**	**2.1**
	P8'	*106.2 (28.2)*	*54.4*	*15.4*	*30.2*	*3.5*	*72.4*	*18.8*	*5.3*
	P2	114.8 (30.6)	52.4	15.9	31.7	1.3	94.3	1.3	3.0
Whole seed based products	P3	37.0 (9.8)	42.4	47.9	9.7	2.8	91.9	0.7	4.7
	P10	45.9 (12.2)	40.5	51.9	7.6	2.3	93.7	0.4	3.6
	P7	**50.6 (13.4)**	**33.3**	**64.4**	**2.3**	**8.0**	**44.7**	**42.7**	**4.6**
	P7'	*53.5 (14.2)*	*34.7*	*62.4*	*3.0*	*6.6*	*45.4*	*41.2*	*6.8*
	P6	**52.8 (13.9)**	**49.3**	**42.3**	**8.5**	**8.6**	**86.6**	**3.4**	**1.4**
	P6'	*61.9 (16.2)*	*59.6*	*29.8*	*10.5*	*13.6*	*82.6*	*2.5*	*1.4*
	P22	77.7 (20.6)	34.7	60.9	4.5	4.4	88.2	3.0	4.4
	P11	91.5 (24.3)	32.2	62.0	5.8	59.5	40.1	0.3	0.1
	P18	91.7 (24.3)	35.9	61.7	2.4	4.5	84.4	1.4	9.8
	P16	116.3 (30.9)	31.8	64.4	3.9	25.2	55.7	7.2	11.9
	P17	132.3 (35.3)	22.3	76.3	1.3	19.0	78.2	1.7	1.0
	P15	138.5 (36.7)	37.0	59.4	3.6	49.6	44.3	4.3	1.8
	P13	144.6 (38.5)	33.2	59.4	7.4	4.5	87.1	0.8	7.7
	P9	**205.1 (54.2)**	**43.1**	**49.7**	**7.1**	**7.1**	**84.6**	**0.8**	**7.6**
	P9'	*137.2 (36.2)*	*44.7*	*45.8*	*9.4*	*3.5*	*91.0*	*0.6*	*4.9*
	P20	245.7 (65.0)	37.5	61.7	0.9	91.4	8.0	0.2	0.4
	P4	292.0 (77.2)	43.4	48.3	8.3	3.3	89.8	1.7	5.2
	P5	292.1 (76.7)	54.8	38.1	7.1	7.1	87.0	1.5	4.4
	P12	365.6 (95.3)	66.1	27.6	6.3	5.3	64.0	26.0	4.7
SD		5.4 (1.4)	2.1	2.1	2.1	1.8	2.2	2.7	2.8

Values in mg/g were calculated using the aglycone molecular weights (254.24, 270.23 and 284.27 g, for daidzein, genistein and glycitein, respectively). According to their isoflavone composition, the dietary supplements were separated in two groups: the glycitein-rich soy germ based products, and the genistein-rich whole seed based products. In each group, the products were ordered by increasing total isoflavone contents (μmoles per g dry weight). Whatever their raw material origin, two batches from 5 out of these products were analysed (the pair is noted Pn, in bold, Pn' in italic). Profiles were given in percentage of the total isoflavone content (calculated with molar values). Conjugation profiles could be related to the process conditions encountered.

largely predominant (45-65% of total isoflavones) [29, 47]. In 16 supplements tested (P3-P7; P9-P13; P15-P18; P20; P22), glycitein represented less than 10.5% of total isoflavone levels, suggesting that these products were issue from whole seeds or cotyledons. In contrast, glycitein level ranged from 24.3% to 35.1% of total isoflavone level in 9 products (P1-P2; P8; P14; P19; P21; P23-P25), indicating that these supplements were issued from soy germ. In this study, we have also shown a high variability in the conjugation profile of soybean isoflavones *(table 1)*. Indeed, the native soybean seeds contain predominantly malonylglycoside isoflavones and a minor percentage of aglycones. Industrial processing can strongly modify isoflavone profile in the end product. For instance, high temperature and pH conditions during processing induce the degradation of the malonyl- and acetyl-conjugated forms leading to β-glycosides [13]. Under more drastic treatments or fermentation processes, almost all conjugated isoflavones were converted into aglycones, and a toasting or roasting process at low moisture increases the acetylglycoside isoflavone concentration [48]. In 15 tested supplements more than 80% of β-glycoside isoflavones were observed *(table 1)*, suggesting that high temperature or pH were used during their processing. The 3 products containing more than 45% of aglycone isoflavones (P11, P15 and P20) were probably submitted to stronger treatments or fermentation processing. The 5 products containing more than 30% of acetylated isoflavones (P1; P19; P21; P23; P24) were probably toasted or roasted at low moisture concentration. Interestingly, the former originated from whole seed, whereas the latter originated from soy germ. This reflects the very different process encountered by these two fractions. Several studies have investigated the role of glycoside *versus* aglycone forms of isoflavones in metabolism and distribution of these compounds on animal and human models [49, 50]. It is generally accepted that aglycone forms are absorbed faster by the gut than glycoside forms [51]. On the other hand, De Pascual-Teresa *et al.* [52] have shown that plasma level of ingested isoflavones depends on the food matrix. Taken together these data, it seems important to provide qualitative and quantitative information concerning the levels of aglycone versus conjugated isoflavones in the dietary supplements.

Table 2. *Soyasaponin A and B contents and composition in 25 soy based dietary supplements.*

		Composition profile					Conjugation profile
		total soyasaponins µmol/g (mg/g)	**soyasaponin A µmol/g (mg/g)**	**soyasaponin B µmol/g (mg/g)**	**%(V+αg)**	**isoflavone /soyasaponin molar ratio (mass ratio)**	**%non-DDMP**
Soy germ based products	P1	15.8 (19.1)	8.5 (12.2)	7.3 (6.9)	28.7	1.1 (0.23)	87.7
	P19	39.5 (46.9)	19.6 (28.1)	19.9 (18.7)	30.9	0.9 (0.21)	93.0
	P21	36.7 (45.2)	21.5 (30.9)	15.2 (14.3)	25.0	1.1 (0.24)	89.0
	P25	24.7 (31.8)	17.2 (24.7)	7.5 (7.1)	28.2	2.5 (0.51)	89.4
	P24	37.1 (45.5)	21.3 (30.6)	15.8 (14.9)	21.9	1.7 (0.37)	100.0
	P23	37.5 (46.5)	22.6 (32.5)	14.9 (14.0)	22.2	1.8 (0.38)	93.1
	P14	**90.5 (110.7)**	**51.5 (74.0)**	**39.0 (36.7)**	**29.3**	**1.3 (0.28)**	**92.9**
	P14'	*62.0 (78.6)*	*40.9 (58.7)*	*21.1 (19.9)*	*29.4*	*1.6 (0.34)*	*96.1*
	P8	**91.1 (113.0)**	**54.9 (78.8)**	**36.3 (34.2)**	**26.5**	**1.6 (0.35)**	**65.8**
	P8'	*78.0 (94.5)*	*42.6 (61.2)*	*35.4 (33.3)*	*25.9*	*1.4 (0.30)*	*66.7*
	P2	48.9 (48.9)	34.0 (48.8)	15.0 (14.1)	27.5	2.3 (0.49)	96.2
Whole seed based products	P3	7.9 (9.2)	3.6 (5.2)	4.3 (4.1)	6.6	4.7 (1.06)	94.7
	P10	6.4 (6.5)	0.9 (1.3)	5.5 (5.2)	5.5	7.2 (1.88)	100.0
	P7	**5.8 (6.8)**	**2.8 (4.0)**	**3.0 (2.8)**	**4.1**	**8.7 (1.96)**	**79.9**
	P7'	*6.8 (8.0)*	*3.2 (4.6)*	*3.6 (3.4)*	*14.9*	*7.9 (1.78)*	*76.1*
	P6	**11.6 (13.1)**	**4.4 (6.3)**	**7.2 (6.8)**	**1.0**	**4.6 (1.06)**	**89.0**
	P6'	*11.9 (13.6)*	*4.8 (6.9)*	*7.1 (6.7)*	*6.4*	*5.2 (1.19)*	*100.0*
	P22	29.9 (32.4)	8.5 (12.2)	21.4 (20.2)	3.4	2.6 (0.64)	80.6
	P11	89.3 (104.5)	41.4 (59.5)	47.8 (45.0)	6.9	1.0 (0.23)	61.1
	P18	55.1 (58.2)	12.8 (18.4)	42.3 (39.8)	6.6	1.7 (0.42)	93.6
	P16	51.3 (55.6)	14.8 (21.3)	36.5 (34.4)	3.1	2.3 (0.56)	96.1
	P17	47.1 (49.0)	9.4 (13.5)	37.7 (35.5)	2.3	2.8 (0.72)	86.9
	P15	112.5 (112.5)	26.4 (37.9)	86.1 (81.1)	4.7	1.2 (0.31)	89.3
	P13	33.0 (35.2)	8.4 (12.1)	24.6 (23.2)	9.0	4.4 (1.09)	86.2
	P9	**149.9 (168.6)**	**55.2 (79.3)**	**94.8 (89.3)**	**8.0**	**1.4 (0.32)**	**100.0**
	P9'	*25.0 (26.4)*	*5.8 (8.3)*	*19.2 (18.1)*	*4.6*	*5.5 (1.37)*	*100.0*
	P20	25.7 (28.5)	8.9 (12.8)	16.7 (15.7)	3.7	9.6 (2.28)	96.1
	P4	64.5 (75.5)	29.9 (42.9)	34.6 (32.6)	9.3	4.5 (1.02)	100.0
	P5	59.3 (68.2)	25.0 (35.9)	34.3 (32.3)	9.2	4.9 (1.12)	96.5
	P12	28.3 (32.9)	12.7 (18.2)	15.6 (14.7)	6.5	12.9 (2.89)	97.8
SD		7.7 -	5.9 (8.5)	5.8 (5.5)	3.8	-	3.5

The products are ordered according to the raw material classification and isoflavone contents established in *table 1*. Considering their high representation (more than 60%) in each groups, values in mg par g are calculated using the molar value of soyasaponin A1 (1436 g) or soyasaponin I (942 g) for groups A and B, respectively. For 5 products, the 2 batches analysed were noted Pn, in bold, and Pn', in italic. The soyasaponin B (V+αg) percentage of total soyasaponins B contents differentiates the soy germ based from the whole seed based products. The aglycones soyasapogenols A, B and the total soyasaponin contents are expressed in µmoles per g dry weight.

The duplicate batches displayed about 30% decrease (P8 vs P8'; and P9 vs P9'), or 17% increase in total isoflavone contents as in P6 vs P6' *(table 1)*. Such order of discrepancies has already been reported between claimed and observed isoflavone contents. In 2003, Bennetau-Pelissero et al. have observed a great variability of isoflavone contents in various french food supplements, as well as significant differences between batches from the same brand [53]. Two major causes are generally underlined: the raw material variability, and the absence of normalization of the isoflavone analysis [21]. In this study, the raw material variability, together with possible process adjustments appears to constitute the major cause of these discrepancies. Nevertheless, no complete raw material change, as soy germ *versus* whole seed, was observed in any product. Changes in the conjugation profile can indicate some heating modifications, as seen in P8 versus P8'. However, in these 5 products, the changes in isoflavones profiles are less important than in isoflavone contents.

Soyasaponin content and composition

The total soyasaponin contents in the 25 dietary supplements ranged from 5.8 µmol/g (P7) to 149.9 µmol/g (P9), with total soyasaponins A ranging from 0.9 µmol/g (P10) to 55.2 µmol/g (P9) and total soyasaponins B ranging from 3.0 µmol/g (P7) to 94.8 µmol/g (P9) *(table 2)*. These results revealed a higher variability in saponin than in isoflavone contents among the products tested. Soyasaponin concentrations were lower than those of isoflavones in 21 products (isoflavone to soyasaponin ratio from 1.2 to 12.9), but similar or slightly higher concentration than isoflavones were observed in 4 products tested (P1, P19, P21, P11 had ratios between 0.9 and 1.1). The soyasaponin profiles also reflected the origin of the raw material used. The products supposed to be issued principally from soy germ due to their isoflavone profiles, contained on average 27% of (V + αg) soyasaponins B, as well as high amounts of soyasaponins A. In contrast, products supposed to be issued from whole

soybean seeds contained lower levels of soyasaponins A, which are more characteristic of the soy germ fraction [54, 55]. Duplicate batches of the same product can exhibit higher variations in soyasaponin than in isoflavone contents: 80% or 30% decrease in P9 versus P9', or P14 versus P14', respectively. Thus, soyasaponin contents are more influenced by the industrial processing condition used or by variations in the raw material. During processing, the 2,3-Dihydro-2,5-Dihydroxy-6-methyl-4H-pyran-4-one (DDMP)-conjugated soyasaponins αg, βg, βa, γg, γa, considered as the naturally occurring saponins in native soybean [28], are mostly converted to their respective non-DDMP counterparts saponins V, I, II, III, and IV, due to the high lability of the DDMP group at high temperature and alkaline pH [48]. These non-DDMP soyasaponins represented on an average 89.8% of the total soyasaponins B in the 25 dietary supplements analyzed. Information concerning their presence and concentration in the soy isoflavone dietary supplements available in the market is of great interest, since the levels of soyasaponins found in the dietary supplements were of the same order as the active doses reported in the litterature [56]. Indeed the daily intake of triterpene saponins used in traditional practice range from a minimum of 60 mg to about 600 mg, what provides an indication of a both safe and effective dosage on the base of traditional practice. Furthermore, it is possible that isoflavones and soyasaponins act through multiple discrete pathways to affect the host health, and that low concentrations of each compound can act synergistically [57]. However, more research is needed to understand the mechanisms of action involved in this interaction.

Quantification of total proteins

The protein contents ranged from 0.4% (P3) to 42.9% (P20) *(table 3)*. Among the dietary supplement tested, 11 products contain less than 10% proteins, whereas four others contain more than 30% proteins, indicating significant differences in protein contents from one product to another, whatever the origin of the raw material used. The native soybean seed contains up to 48% proteins, consisting in a mixture of α-, β-, and γ-conglycinins, glycinin, and other globulins, ranging in molecular weight from 140 to 300 KDa and differing in physicochemical properties according to their structure [58]. The levels of proteins in the dietary supplements were highly dependent on the processing conditions. High temperature and pH increase protein solubilization and therefore increase protein recovery, what also promotes saponin extraction. A water washing treatment concentrates the proteins by removing very polar phytochemicals and oligosaccharides, whereas an ethanol washing treatment substantially decreases the concentration of these polar lipophilic phytochemicals [48]. As reported in a recent study [13], soy molasses, which were earlier considered only for their economical and caloric properties, are now used for the production of isoflavone ingredients, resulting in products presenting high protein levels. In contrast, an aqueous-alcohol washed isoflavone extract contains low protein concentration. Soy proteins have been the focus of many investigations concerning their health promoting properties. It is widely recognized and confirmed by numerous human studies that soy protein consumption results in significant lowering of serum cholesterol levels [59-61], as well as in a reduction in systolic and diastolic blood pressure in persons with hypertension [62]. On the other hand, it has been observed that isoflavone enriched products consumed by premenopausal women lowered LDL cholesterol by 7.6 to 10% [63], and play an active role in the prevention of cardiovascular diseases [64]. Thus, soy proteins in soy isoflavone concentrates could provide an additional effect on the serum lipid profile. Today, it remains not clear if the hypocholesterolemic effects of a soy-based diet are dependent of the proteins themselves, through the action of active oligopeptides produced in the intestinal tract, or if these benefits may be attributed to oestrogenic activity of the isoflavones [65]. In addition, other effects of soyasaponins on serum lipid profiles have been reported to be dependent on the presence of dietary proteins [23], suggesting a possible interaction between saponins, glycinin, and β-conglycinin through hydrophobic interaction, hydrogen bonding or ionic bonding [66]. However in this study we have not determined specific peptides presenting health properties.

Table 3. *Total protein and lipidic fractions in 25 soy isoflavone dietary supplements.*

	Product	Total proteins g/100g DW	Total lipids g/100g DW
Soy germ based products	P1	16.1	10.2
	P19	33.5	11.6
	P21	36.7	14.5
	P25	3.9	15.3
	P24	1.4	7.4
	P23	8.9	21.8
	P14	21.3	10.5
	P8	10.8	15.2
	P2	5.7	3.8
Whole seed based products	P3	0.4	1.6
	P10	6.7	2.3
	P7	18.4	19.4
	P6	6.0	19.4
	P22	34.9	4.1
	P11	7.5	4.1
	P18	26.1	4.6
	P16	12.1	10.2
	P17	11.5	5.3
	P15	10.5	10.4
	P13	1.4	8.4
	P9	6.4	3.6
	P20	42.9	3.9
	P4	3.4	6.6
	P5	2.3	5.6
	P12	3.1	3.0
SD		1.2	2.2

The products are classified and ordered as in *table 1*. Protein contents were determined by the DUMAS method, and lipid contents were calculated after *n*-hexane extraction under reflux. The contents are expressed in g per 100 g DW.

Determination of fatty acids

Most of the 25 dietary supplements analyzed were defatted products, since only 11 products contained more than 10% lipids *(table 3)*. Among these 11 non-defatted products, 4 were too waxy for their fatty acid profile determination. Consequently, only 7 products were used for fatty acid profile determination *(table 4)*. The average composition of fatty acids in the non-defatted dietary supplements issued from soy gems was 19.9% palmitic acid (16: 0), 10.8% stearic acid (18: 0), 9.9% oleic acid (18: 1), 48.3% linoleic acid (18: 2), and 11.1% linolenic acid (18: 3), reflecting typical composition of refined soygerm oil [37]. The fatty acid profile of the whole seed is generally reported to be higher in oleic (24%) and linoleic acid (54%), and lower in palmitic (11%) and linolenic acid (7%) [36]. This indicates that some soy isoflavone dietary supplements are particularly concentrated in fatty acids, such as P8 which contains 79.3 ± 3.7 mg/g of total fatty acids, while others are totally devoided of lipids. Linoleic acid (18: 2) is the predominant plant-derived dietary polyunsaturated fatty acid, and it was found predominantly in all the analyzed products. The high contents of fatty acids in some of the dietary supplements analyzed in this study may confer additional properties to

Table 4. *Fatty acid composition of soy isoflavone dietary supplements.*

Product	Total oil %	Palmitic acid 16:0 (mg/g)	Stearic acid 18:0 (mg/g)	Oleic acid 18:1 (mg/g)	Linoleic acid 18:2 (mg/g)	Linolenic acid 18:3 (mg/g)	Total fatty acids (mg/g)	SFA (% total FA)	PUFA (% total FA)
P1*	10.2%	14.6 ± 0.9	11.1 ± 0.7	5.4 ± 0.2	23.3 ± 1.1	3.5 ± 1.1	57.9 ± 0.7	44.4%	46.3%
P19*	11.6%	7.2 ± 0.4	3.1 ± 0.1	5.0 ± 0.1	25.0 ± 0.5	5.9 ± 0.1	46.2 ± 1.1	22.3%	66.9%
P21*	14.5%	10.3 ± 1.7	5.5 ± 0.4	8.5 ± 0.9	34.7 ± 5.3	8.4 ± 1.2	67.4 ± 9.7	23.4%	63.9%
P23*	21.8%	16.4 ± 1.4	12.2 ± 0.9	5.0 ± 0.1	19.8 ± 1.3	4.1 ± 0.4	57.5 ± 4.0	49.7%	41.6%
P14*	10.5%	8.2 ± 0.2	4.6 ± 0.1	6.6 ± 0.1	28.5 ± 0.5	7.0 ± 0.1	54.9 ± 0.8	23.3%	64.7%
P8*	15.2%	14.5 ± 1.0	3.5 ± 0.1	6.3 ± 0.4	43.9 ± 1.8	11.1 ± 0.4	79.3 ± 3.7	22.7%	69.4%
P7**	19.4%	8.1 ± 1.2	5.6 ± 0.6	0.7 ± 0.1	0.9 ± 0.3	0.10 ± 0.02	15.4 ± 1.5	89.0%	6.5%

n = 2, FA : Fatty Acid, SFA: Saturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids. *: soy germ based product ; **: whole seed based product. These only 7 products had enough and significant lipids amounts (cf. *table 3*).

these products, as polysinsaturated fatty acids can have heath promoting activity, even at very low doses [67]. It has been shown in a recent study that postmenopausal women suffering from hot flushes and ingesting two capsules per day providing 60 mg of isoflavones and also two capsules containing a polyunsaturated fatty acid supplement for 24 weeks showed highly significant reduction in the number of hot flushes. These results indicated that polyunsaturated fatty acids may contribute to the effects generally attributed to the isoflavones at the same level of intake.

Quantification of α-galactooligosaccharides

Native soybean seeds contain high concentrations of two α-glycosidic galactooligosaccharides, raffinose (0.1-0.9%) and stachyose (1.4-4.1%) [68]. The total α-galactooligosaccharide contents in the 25 soy isoflavone dietary supplements ranged from non-detected to 88.8 mg/g, reflecting a high variability of α-galactooligosaccharide concentration in the products tested *(table 5)*. Stachyose, which structurally contains a fructose, a glucose, and a galactose was more concentrated than raffinose which contains one more galactose, in all the products analyzed. For a long time, these compounds were considered undesirable because some flatulence problems associated with human consumption of soy products were attributed to oligosaccharides. However, recent reports have shown that soy oligosaccharides do not alter significantly the nutrient digestibility, and in contrast they may constitute a powerful prebiotic [69]. Indeed, many products containing stachyose and raffinose are commercialized for their prebiotic properties. Prebiotics may enhance the effects of isoflavones by converting the inactive isoflavone glycosides to the biologically active aglycones [70], or by influencing the biosynthesis of equol, an interesting daidzein metabolite which is more estrogenic and more antioxidative than daidzein [71, 72]. The intestinal conversion of daidzein to equol has been the focus of great interest, and dietary carbohydrates have been indicated as a factor that may determine equol production [73, 74]. Soy oligosaccharides have an impact on the metabolic activity of intestinal microflora, and are implicated in the prevention of colon cancer. These complex sugars stimulate the growth of *Bifidobacterium* and *Lactobacillus* species in the large intestine, and increase the production of short chain fatty acids [75]. Since the active phytoestrogenic isoflavones present an aglycone structure generated by the bacteria of the large intestine, ingestion of soy oligosaccharides may influence the plasmatic concentration of isoflavones [76]. It has also been shown *in vivo* that a diet containing less than 22.4 g of stachyose/kg body weight and less than 2 g of raffinose/kg did not alter digestibility or increase flatulence, indicating that a low intake of soy oligosaccharides did not exert undesirable effects compared with a high intake [77]. In another recent study, patients with seasonal allergic rhinitis treated with 600 mg of soy sauce polysaccharides showed a significant reduction in the total symptom score [78]. Thus, the presence of raffinose and stachyose, even in low quantity in the dietary supplements may influence the biological effects attributed to isoflavones, or exert independent biological activity. Their amount in the commercial products need to be determined.

Conclusion

In this study, we have shown that soy-derived dietary supplements contain highly variable amounts of isoflavones, soyasaponins, proteins, fatty acids, and α-galactooligosaccharides. The content and composition of these compounds depend on several factors, including the nature of

Table 5. *Quantification of α-galactooligosaccharides in 25 soy isoflavone dietary supplements.*

	Product	Stachyose (mg/g)	Raffinose (mg/g)	Total α-galactooligo-saccharides (mg/g)
Soy germ based products	P1	29.0	7.0	36.0
	P19	60.3	15.1	75.4
	P21	71.2	16.8	88.0
	P25	1.2	1.2	2.4
	P24	15.7	5.6	21.3
	P23	ND	2.0	2.0
	P14	63.8	15.9	79.7
	P8	69.9	16.8	86.8
	P2	ND	ND	ND
Whole seed based products	P3	3.6	2.1	5.7
	P10	3.6	0.8	4.5
	P7	48.8	14.8	63.5
	P6	3.9	2.4	6.4
	P22	50.9	10.3	61.2
	P11	0.6	0.6	1.2
	P18	47.3	14.7	62.0
	P16	7.5	2.2	9.7
	P17	11.0	3.1	14.1
	P15	5.5	2.1	7.7
	P13	ND	ND	ND
	P9	0.4	0.5	0.9
	P20	9.6	1.8	11.4
	P4	3.4	1.6	5.0
	P5	0.9	0.7	1.6
	P12	4.4	1.5	5.8
SD		5.2	5.6	7.3

The products were ordered as defined in *table 1*. (ND : below detection limit).

the raw material and the industrial processing conditions that may influence the extraction of particular phytochemicals, or modulate their conjugation profile. Even at low concentration, these compounds may have significant health consequences if their bioavailability is very high or if they act synergistically or as antagonists. Consequently, the determination of a precise and global profile of phytochemicals in the soy-derived supplements as well as studies concerning the biological effects of their interaction, are needed in order to establish right claims and avoid unexpected side effects associated with their use.

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