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ELISA as a new method to measure genistein and daidzein in food and human fluids

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Abstract

Widely distributed in the plant kingdom, phytoestrogens, like soy isoflavones are found in plant protein extracts at varying levels depending on culture conditions and cultivars. Their increasingly used in human, as natural estrogens and their varying levels in raw materials raise the question concerning the measurement of isoflavones both in food or food supplements as well as in human fluid. To validate our new ELISA technique, isoflavones measurements in food-supplements, in soy food and in human fluid from volunteers participating to a kinetic study or a survey, were done. Our method was also compared with other physico-chemical techniques in an inter-laboratory assay (23 laboratories). In conclusion, our new ELISA technique is reliable, sensitive, cheap, rapid and can be used either for a great number of human fluid analysis, for crude matter or food analysis as long as the appropriate extraction technique is performed prior to ELISA procedure.

Keywords: ELISA; Soybean isoflavones; Human plasma; Human urine; Soy-food; Soy based food-supplements

1. Introduction

Phytoestrogens are estrogeno-mimetic molecules of plant origin (Bradbury & White, 1954). Isoflavones, contained mainly in leguminosae for human diet like soy and kudzu, or for animal diets, such as clover, alfalfa and beans, are widely used today as estrogen supplements (Hsu, Shen, Hsueh, & Yeh, 2001; Setchell et al., 2001). These supplements are prescribed to menopausal women with estrogen deficiency in order to counteract mood changes and hot flashes classically occurring during this period of life (Grainge, Coupland, Cliffe, Chilvers, & Hosking, 2001). Manufacturers have reported other effects such as reduction of vaginal dryness and breast pain (Whitten & Naftolin, 1998). Other health allegations are sometimes mentioned on tablet packaging. These are protection against osteoporosis and against cardiovascular diseases. Whereas not demonstrated in humans, improvement of these diseases has been obtained in animal models, notably rats and monkeys (Picherit, Bennetau-Pelissero et al., 2001; Setchell & Cassidy, 1999). Beneficial effects have also been reported in humans although far from being always conclusive, and frequently controversial. On cardiovascular diseases, many studies reported a decrease in plasma total cholesterol and LDL cholesterol but this reduction is always of low value (between 7 and 12% only). Moreover, some studies were performed using soy protein and not isolated isoflavones and these proteins were used for a short period in substitution for another protein (Merz-Demlow et al., 2000; Potter, Baum, Teng, Stillman, Shay, & Erdman, 1998; Wangen, Duncan, Xu, & Kurzer, 2001). In some cases, soy protein were used as supplement but this time again

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the decrease in total and LDL cholesterol was discrete (7–10%) (Hermansen, Sondergaard, Hoie, Carstensen, & Brock, 2001; Washburn, Burke, Morgan, & Anthony, 1999). When different levels of isoflavones were tested the effect were not always different (Jenkins et al., 2002) and thus the impact of pure isoflavone preparations remain to be proved. On osteoporosis, soy protein was shown to prevent bone loss on a 6-month exposure (Potter et al., 1998) but no effects from isolated isoflavones have been reported so-far. The effect of soy or isoflavone consumption on cancer is still unclear. Epidemiological data show that Asian diets reduce cancer risks (Goodman, Wilkens, Hankin, Lyu, Wu, & Kolonel, 1997) but the confounding factors due to the large differences between Asian and Western diets prevent us from making a conclusion on the effect of dietary isoflavones although pharmacological doses give interesting results. Indeed, anti-tyrosine kinase effect or anti-angiogenic properties of certain isoflavones can be involved in cancer prevention as well as in the protection against other pathologies (Joussen, Rohrschneider, Reichling, Kirchhof, & Kruse, 2000). In addition, the equol production and excretion seems to have to be taken into account in the evaluation of cancer risks in soy consumers (Duncan, Merz-Demlow, Xu, Phipps, & Kurzer, 2000). This, has to be taken into account when effects of isoflavones are compared between animals and humans.

In France, more than 30 food-supplements for menopausal women have been commercialised thus far. Most of them contain soy, and a few contain additional plants, such as clover, alfalfa, yam, hops or cimicifuga... The estrogenic effect of soy isoflavones, namely genistein and daidzein, as well as that of the daidzein metabolite equol, has been recognized for quite a long time and have been widely explored both in animal (Kaziro, Kennedy, Cole, & Southwell-Keely, 1984; Kondo, Suzuki, Ikeda, & Umemura, 2002; Shemesh, Lindner, & Ayalon, 1972; Shutt & Cox, 1972; Thompson, Lasley, Rideout, & Kasman, 1984; Verdeal, Brown, Richardson, & Ryan 1980) and human tissues (Martin, Horwitz, Ryan, & McGuire, 1978; Welshon, Murphy, Koch, Calaf, & Jordan, 1987). This time again results can be controversial with studies reporting protective effects on cancers (Fritz, Eltoum, Cotroneo, & Lamartinière, 2002; Lamartiniere, Moore, Brown, Thompson, Hardin, & Barnes, 1995) when others are more ambiguous (Cohen, Zhao, Pittman, & Scimeca, 2003; Hilakivi-Clarke, Cho, Onojafe, Raygada, & Clarke, 1999; Shirai, Asamoto, Tkahashi, & Imaida, 2002). This effect is clearly influenced by the doses.

Soy-food for both animal and human consumption, usually also contains large amounts of phytoestrogens (Anderson & Wolf, 1995; Aussenac, Lacombe, & Dayde, 1998). Up to recently isoflavones were classically assayed using very accurate physico-chemical methods including HPLC and GC coupled to various detection methods (Axelson, Sjövall, Gustafsson, & Setchell, 1984; Jones, Price, & Fenwick, 1989; Setchell, Welsh, & Lim 1987). These methods are not always sensitive enough to assay plasma samples. A few immunological assays for isoflavones have been developed and presented so far, but they do not seem to be used currently (Hampl, Lapèík, Wähälä, Starka, & Adlercreutz 1998; Lapèík, Hampl, Al-Maharik, Salkla, Wähälä, & Adlercreutz, 1997; Lapèík, Hampl, Hill, Wähälä, Al Maharik, & Adlercreutz, 1998; Wang, Tanaka, Han, & Cheng, 1994). They are less convenient than ELISAs because they are based on the use of radioactive material, although they are currently more sensitive.

Because phytoestrogen effects are clearly linked to ingested doses, it was deemed interesting to develop specific ELISAs to measure genistein, daidzein and equol plasma levels, in order to correlate isoflavone ingestion, circulating levels and effects. This has already been done previously in animal studies in collaboration with other scientific teams (Blair et al., 2002; Picherit et al., 2000; Picherit, Bennetau-Pelissero et al., 2001; Picherit, Chanteranne et al., 2001). In this study, a new ELISA technique is presented and validated by comparison with other physico-chemical methods. A survey of isoflavone plasma levels in 207 healthy French menopausal women under a low isoflavone concentrated soy supplement (Biopause®) is also presented. The survey, which corresponds to measurements randomly distributed during the day, reflects the mean isoflavone plasma levels of a given population. This concept could be interesting when the evaluation of the exposure of a global population is considered. The technique is then shown to be reliable for multiple sample analysis and sensitive enough to discriminate between low ingested isoflavone doses. The ELISA technique is used in women to measure isoflavones in plasma and urine sampled in a kinetic approach. The technique is also used to assay soy food and food supplement based on soy.

2. Material and methods

2.1. Origin of the analysed samples

2.1.1. Soy-based food supplements

The commercial soy-based food supplements were obtained from chemists or supermarkets depending on the distribution circuits. For each trademark a tablet was used for the analysis and the measurements were performed in triplicate. None of the food supplements assayed here contained other plant except soy.

2.1.2. Soy food

They were obtained either in supermarkets or in special organic food shops. This time again for each food

category only one item was considered but its measurement was performed in triplicate.

2.1.3. Women's plasma and urine samples

Two studies are presented here: a kinetic study and a survey. In each case mean results are obtained on two dilutions each in duplicates.

In the kinetic study, three female volunteers ingested a soybean extract containing 100 mg of total isoflavones expressed in equivalent aglycone (2/3 genistein and 1/3 daidzein). Genistein and daidzein were incorporated as a powder to two yoghurts and two biscuits and absorbed in a single intake. Blood samples were collected on heparinized syringes 0, 4, 6, 8, 10 and 24 h after intake. After plasma separation and collection, it was stored at −20 °C until the extraction procedure is performed. Urine samples were collected 0, 6, 10, 24 and 32 h after ingestion. Samples were simply stored at −20 °C until the extraction procedure is performed. The volunteers did not have soy, leguminosae, chocolate, tea or coffee for 3 days before starting the experiment.

The survey was performed on 207 volunteers between 43 and 70 years of age (55.8±4.9, median: 55). All were healthy menopausal women having had Biopause® treatment (2.45 mg/tablet of total isoflavone expressed in equivalent aglycone) for at least 6 months except for the controls. They weighted between 45 and 120 kg (61.4±10.9, median: 60). The women considered in this study were prescribed 0, 2 (4.9 mg) or 4 tablets (9.8 mg) a day, according to the severity of hot flashes and the effect of the treatment thereon. They take their treatment in the morning or in the evening according to their choice. During the study the volunteers followed their own habitual diet and agreed to indicate the major tendency of their regime, filling in a short specific questionnaire (see Fig. 1). For all anonymous women the following information was collected: health status, age, weight, date of blood sampling, time of blood sampling, and general diet tendencies. Twenty-five women were removed from the study because their answers to the questionnaire were incoherent, or because they changed their dietary habits the day before sampling or because they had had antibiotics within 3 weeks before sampling. The women retained for this study had no major health problem as could be judged by their answer to the medical inquiry. None of them had answered yes to any question (see Fig. 1). Plasma samples were collected as mentioned previously according to the specific medical rules. They were collected all day long.

2.2. Extraction procedures

2.2.1. Isoflavone extraction from plasmas

Genistein, daidzein and equol contained in 500 μl of plasma were first hydrolysed using β glucuronidase aryl sulfatase from *Helix pomatia*. For this hydrolysis 2 ml of a 10 μl/ml solution of β glucuronidase aryl sulfatase from *Helix pomatia* (Roche, Ref 127 698) in acetate buffer (pH5; 0.01 M) is added. The mixture is incubated for 48 h at 37 °C under slow agitation. The hydrolysis is monitored using external standard run in parallel. These standards contain either 1 mg/ml genistin or 1 mg/ml daidzin the glycoside forms of genistein and daidzein, respectively. The ELISA technique is used to check the recovery of the external standard hydrolysis. A liquid–liquid extraction is performed three times with 5 ml of acid–ethyl acetate. Organic extracts are pooled and evaporated to dryness before dissolution in 500 μl assay buffer (PBS–T–PS–DMSO–PBS containing 0.1% porcine serum, 0.05% Tween 20 and 1% DMSO) and sonicated to ensure complete dissolution. Sonication is performed using a Vibra-Cell 75021 Ultrasonic Processor from Bioblock scientific. Samples are treated for 2 min at room temperature (3 W). Extracts are stored at −20 °C until assay. Three external standards of extraction containing genistein and daidzein are run in parallel to check for extraction recovery.

The hydrolysis and extraction procedures were initially checked for recovery using concentrated solution of genistein, daidzein and equol (1 mg/ml) using HPLC coupled to a DAD detector with C18 ODS 2 column and acetonitryl–water gradients. It was shown that the recovery measured on five different samples for each compound ranged between 98 and 100% for hydrolysis as well as for extraction.

2.2.2. Extraction procedure for soy based tablets

The extraction procedure for tablets was performed on one tablet. It was dissolved in 100 ml of distilled water and 500 μl of the suspension is sampled while stirring for the following steps of hydrolysis and extraction. These hydrolysis and extractions are performed as mentioned earlier and the recoveries for each step are monitored as previously mentioned.

2.2.3. Extraction procedure for soy foodstuff

The extraction procedure in soy-food varied according to the food processed but is always performed in triplicate. The procedures followed are indicated later for each foodstuff category. In each case, the same hydrolysis and extraction control are performed.

2.2.4. Extraction of liquid food-stuff

Milk, yoghurts, desserts and dried powdered milk, reconstituted with water are all considered as liquid foodstuff. In all cases, the extraction procedure is applied to 1 ml of solution, diluted 1:100 in distilled water. Hydrolysis and extraction are performed as mentioned earlier. The external standards for hydrolysis and extraction are run in parallel.

2.2.5. Fatty food-stuff extraction

Cheese, cream and tofu are considered as fatty food-stuff. One gram is dissolved in 100 ml distilled water heated at 90 °C for 10 min while being stirred; 1 ml is then used for the following steps. Equol is added as an internal standard just before 2 ml of acetate buffer (pH5; 0.01 M) and β glucuronidase aryl sulfatase to perform the hydrolysis as previously described. The dry extract obtained after acid ethyl–acetate extraction is dissolved in hexane, and passed through a C18 Sep Pack cartridge. After rinsing three times with hexane, the genistein, daidzein and equol are eluted with methanol. Methanol is evaporated to dryness and the extract is dissolved in assay buffer PBS–T–PS–DMSO. The external standards for hydrolysis and extraction are run in parallel.

Code :
Date :
Age :
Weight :

Dietary Questionnaire for women under Biopause

Diet characteristics:
How many meals do you have per day :

Types of food	**Intake frequency**						
	Less than once a week	once a week	2 to 5 times a week	once a day	twice a day	3 times a day	More
Vegetables	☐	☐	☐	☐	☐	☐	☐
Paste or carbohydrates	☐	☐	☐	☐	☐	☐	☐
Fruits	☐	☐	☐	☐	☐	☐	☐
Meat	☐	☐	☐	☐	☐	☐	☐
Eggs	☐	☐	☐	☐	☐	☐	☐
Fish	☐	☐	☐	☐	☐	☐	☐
Cofea	☐	☐	☐	☐	☐	☐	☐
Tea	☐	☐	☐	☐	☐	☐	☐
Milk products	☐	☐	☐	☐	☐	☐	☐
Milk products + ferments	☐	☐	☐	☐	☐	☐	☐
Wine	☐	☐	☐	☐	☐	☐	☐
Fruit juice	☐	☐	☐	☐	☐	☐	☐
Soy and Soy based products	☐	☐	☐	☐	☐	☐	☐

For how long do have this type of diet ? ___days ___months ___years

How would you characterised your diet ? :
Omnivorous ☐ Vegetarian ☐ Macrobiotic ☐

And more precisely:

Thinness diet	☐	Diet without cholesterol	☐
Diet without salt	☐	Fitness diet	☐
Organic diet	☐	No specific diet	☐
Diet without milk	☐	other :	

Medical characteristics :	yes	no	sometimes
Do you experience liver pain?	☐	☐	☐
Do you experience pancreas pain?	☐	☐	☐
Do you take laxative including plants?	☐	☐	☐
Do you take sleeping pills, anxious pills, mood regulators?	☐	☐	☐
Did you take antibiotics recently?	☐	☐	☐

Did you change you habits yesterday ? (the day before blood sampling)
(exotic meal, no meal ...) ?
yes ☐ no ☐
If yes what did you do? :

Fig. 1. Questionnaire voluntarily filled in by women under Biopause participating to the study.

2.2.6. Lecithin extraction

The procedure is the same as that previously described but solid lecithin is first dissolved into 10 ml hexane. Acetate buffer is added to 1 ml of the hexane solution. A liquid–liquid extraction is performed to extract the hydro-soluble compounds and the procedure, including the enzymatic hydrolysis, is then applied to the aqueous phase. The external standards for hydrolysis and extraction are run in parallel.

2.3. Assay procedure

The assay procedure was previously described by Le Houérou, Bennetau-Pelissero, Lamothe, Le Menn, Babin, and Bennetau (2000) for genistein and daidzein and by Bennetau-Pelissero Le Houérou, Lamothe, Le Menn, Babin, and Bennetau (2000) for equol. These works describe the synthesis of the haptens, the coupling to proteins (Bovine Serum Albumin—BSA and Thyroglobulin—Thyr) and the production of the specific antibodies. They give the sentitivity of the methods. In details, coating of the wells is performed with the Thyr–hapten conjugates (200 µl/well) in solution in carbonate buffer (pH 9.6, 0.05 M) at 4 °C, overnight. The same hapten is used for coating as for immunization, i.e. the assays are hapten homologous. Concentrations of conjugates and antibodies are listed in previous articles (Le Houérou et al., 2000; for genistein and daidzein and by Bennetau-Pelissero et al., 2000 for equol). The wells are then saturated with PBS–T–PS–DMSO at 37 °C for 30 min. Plates are rinsed three times with PBS–T–DMSO (PBS, 0.05% Tween 20, 1% DMSO). Serial dilutions of the analyte in PBS–T–PS–DMSO are prepared as standard curves and 100 µl/well are added to the plate. Specific antibodies are then added (100 µl/well). The incubation lasts for 2 h at 37 °C. The plates are washed three times with PBS–T–DMSO. Then 200 µl/well of the second antibody are added in PBS–T–PS–DMSO. The incubation is performed at 37 °C for 30 min. To measure peroxidase activity, 200 µl/well of substrate solution containing 0.005 M *o*-phenylenediamine (10 mg in 20 ml) and 0.025% H_2O_2 30% (5 µl in 20 ml) in citrate–phosphate buffer (pH 5.0, 0.15 M) are added. The reaction takes place at room temperature for 30 min and stopped with 50 µl/well of H_2SO_4 4 M. ODs are read at 490 nm. The standard curves obtained using synthetic compounds synthesized as described by Pelissero, Bennetau, Babin, Le Menn, and Dunoguès (1991) counts 12 points in duplicates with a two-fold increase between concentrations. The inter-assay variation is 13.1% for genistein, 12.8% for daidzein and 13.6% for equol measured comparing the same sample on 10 different plates. The intra-assay variation is 4.8, 5 and 5% for genistein, daidzein and equol assay, respectively measured on the same sample assayed 12 times on the same plate. The samples are run randomly on plates together with an assay control sample (same sample on each plate). The sensitivity of the assays given as the mid point of the standard curve is 40 ng/ml for daidzein, 15.6 ng/ml for genistein and 10 ng/ml for equol. The detection limit is therefore 10 ng/ml for daidzein, 3.9 ng/ml for genistein and 2.5 ng/ml for equol. All samples have to be diluted at least at 1:5 in order to avoid non-specific cross-reaction with matrix residues.

2.3.1. Specificity tests

They were performed against isoflavones from the same family as well as against flavones and a steroid. The results are given in Table 1.

2.4. Assay comparison

Acatris Holding BV organized a comparison of methods in 2001, in co-operation with TNO Nutrition

Table 1
Specificity tests of the antibodies used in this study

Compounds tested	% Of cross reaction		
	Anti-equol	Anti-genistein	Anti-daidzein
Daidzein	*0.12%±0.03*	*2.05%±1.2*	
Genistein	*0.015%±0.004*		*5.22%±10*
Equol		*0.062%±0.02*	*0.19%±0.05*
Formononetin	*0.013%±0.005*	*2.08%±1.1*	*51.5%±10*
Biochanin A	*0.025%±0.005*	*53%±12*	*1.78%±0.5*
O-desmetyl-angolensin	0.05%±0.006	<0.015	<0.016
Flavone	<0.028	0.06%±0.002	<0.016
Quercetin	<0.028	1%±0.4	<0.016
Apigenin	<0.028	1.345%±0.002	<0.016
Chrysin	<0.028	5.25%±1.2	<0.016
Puerarin	<0.028	<0.015	0.64%±0.012
Estradiol	<0.028	<0.015	<0.016

Results are expressed as percent cross-reactivity values (IC_{50} of parent compound/IC_{50} of tested compound)×100±S.D. calculated on three tests. Values in italic were already published in Bennetau et al. (2000) and Le Houérou et al. (2000).

and Food Research (Zeist, The Netherlands) (Verbruggen & van Rooijen, 2001). Participants were commercial, academic and company laboratories: 12 laboratories from Europe, 7 laboratories from USA/Canada, 3 laboratories from Japan/Australia. Twenty-three laboratories participated to the tests. Nineteen laboratories used HPLC/UV, 2 laboratories used HPLC/alternative detection, 1 laboratory used ELISA and another used capillary electrophoresis. Each laboratory received soy germ: 1.5–2.5%, soy germ extract 10–12% and soy extract 40% all in powder form, in duplicate. Samples were homogenized prior to division into sub samples. Homogeneity was confirmed using the *F*-test. Each participant was asked to analyse the six samples according to their current method. One of these laboratories did not send its data back.

2.5. Statistical analysis

For the ring test analysis, the analytical data were statistically evaluated according to ISO 5725 using the statistical program *Ringreport*. Outliers (accidental errors) in differences between duplicate samples were identified, as well as outliers in averages (systemic errors), using the Grubbs outlier test. The statistical evaluation was then repeated without the outliers. For the survey study, the statistical analysis was performed using SAS software and applying the Student–Newman–Keuls analysis because the number of subjects in each group was quite different and because the groups did not follow the normal law and the variance were compared by ANOVA. The significance threshold was considered when it reached 5%.

3. Results

3.1. Assay comparison

Table 2 presents data obtained from the Acatris ring test presented in 2001. Initially, data from all laboratories except ours were presented in acetyl, malonyl and glycoside forms. Our method, which implies a total hydrolysis of all the conjugated forms prior to ELISA analysis could not be compared relevantly. To allow comparison, a conversion of conjugates into aglycone forms was performed. Table 2 show that our results (line 9) are reproducible and in accordance with results from other laboratories, except for sample 5 and 6 which are a bit too high.

3.2. Isoflavone in diet supplements commercialised in France

Table 3 presents the isoflavone content measured by ELISAs in 22 soy-based food-supplements commercia-

Table 2
Assay comparison between 22 laboratories with data converted from the Acatris ring test (2001)

Laboratories number	Samples 1–2				Samples 3–4				Samples 5–6			
	Genistein		Daidzein		Genistein		Daidzein		Genistein		Daidzein	
1	5.59	6.59	6.59	7.26	14.87	8.45	31.34	31.46	178.13	181.20	128.83	129.17
2	3.66	4.05			13.67	6.70	30.60	26.03	158.48	148.29	116.16	108.49
3	0.54	0.80	6.94	7.09	14.20	7.51	40.28	42.06	166.54	166.22	146.69	163.78
4	0.21	0.15	4.61	4.61	8.55	104.72	23.50	98.25	134.18	4.65	125.51	23.16
5	2.14	3.04	3.55	3.34	16.48	16.48	17.61	18.31	160.90	165.64	95.59	97.96
6	0.29	0.17	1.96	1.84	4.47	2.33	9.46	8.59	31.47	31.02	39.90	39.96
7	1.08	1.29	6.75	7.53	11.64	8.80	27.14	30.77	164.05	164.24	115.57	116.20
8	0.88	1.24	6.38	7.13	14.34	8.72	29.07	32.09	31.49	29.63	65.10	61.44
9	2.14	2.87	6.76	7.20	15.04	12.85	31.53	44.20	224.67	211.49	171.82	164.14
10	1.53	2.25	7.62	8.85	14.20	10.92	33.64	43.46	98.16	68.88	103.28	100.05
11	2.05	3.08	4.88	6.31	20.84	15.61	27.40	29.02	59.26	60.75	59.43	63.33
13	0.79	0.68	5.60	6.15	10.44	5.87	27.17	30.76	114.37	109.27	105.74	102.87
15	0.80	1.11	6.80	7.42	9.81	9.07	31.92	34.24	139.25	143.15	122.35	123.61
16	0.27	0.19	5.50	5.32	12.80	5.73	29.56	27.49	164.73	165.38	129.56	128.93
17	1.86	2.33	5.88	5.77	18.66	17.03	26.03	23.67	183.72	177.15	143.55	138.82
18	2.47	2.38	4.94	5.52	22.88	14.91	24.26	27.68	63.10	65.25	82.13	82.11
19	1.38	1.44	12.34	11.34	9.17	10.56	43.18	47.94	17.12	28.84	24.69	22.29
20	1.39	2.27	8.11	10.17	15.91	12.14	30.79	34.70	58.15	63.36	57.04	61.11
21	4.99	5.48			22.92	6.39	26.63	28.43	121.40	115.10	103.30	90.73
22	6.55	8.35	9.40	9.50	42.16	41.35	62.70	60.93				
23	0.44	0.31	5.74	5.90	7.77	5.86	12.78	12.16	11.26	11.23	14.17	17.53
24	1.21	1.20	6.11	5.69	13.15	10.93	24.41	30.58	58.48	55.71	59.05	59.15
Number of laboratories	22	22	20	20	22	22	22	22	21	21	21	21
Mean	1.92	2.33	6.32	6.70	15.18	15.59	29.14	34.67	111.38	103.16	95.69	90.23
Average of physico-chemical techniques	1.91	2.30	6.30	6.67	15.19	15.72	29.02	34.02	105.61	97.75	91.88	86.53

lized in France. From this table, it can be seen that content can vary greatly according to the brand. Following the prescription, women will take from 2.4 mg of total isoflavone per day up to 92.8 mg of total isoflavone per day. Looking closer, Biopause® is one of the lowest concentrated preparations on the market, with 4.9 mg of total isoflavone prescribed per day. According to the present measurements, only few preparations are really in accordance with the amount claimed on the packaging, when the latter is specified.

3.3. Isoflavone intake with French soy-food consumption

Table 4 gives the amount of genistein and daidzein found in French soy-food and the equivalent intake following consumption of a normal portion. Intake is significant, except for lecithin and soy sauce, and nearly always higher than that corresponding to the prescription of Biopause®.

3.4. The kinetic study

The results obtained from the kinetic study are presented in Fig. 2. It is noticed that after an increase in plasma isoflavones, levels return progressively to low levels. The same phenomenon is observed for the urinary profiles although the levels are much higher and the peak is delayed. The genistein plasma levels peak at a mean of 1.127 ± 345 ng/ml and for daidzein the mean peak is at 607 ± 149 ng/ml. Urinary level are much higher with a mean peak level for genistein at $31{,}214 \pm 6432$ ng/ml and for daidzein at $28{,}752 \pm 7877$ ng/ml. Plasma C_{max} were observed between 6 and 8 h after intake whereas urine C_{max} were observed on the samples collected 10 h after ingestion. None of the women were found to be equol producers in this study.

3.5. The survey

From the questionnaire filled in by the volunteers, four different diet tendencies arose. Forty volunteers were considered to be soy-eaters (S) and declared consuming soy-food at least once a day. Seventy-two were omnivorous (O), 58 ate yoghurts containing ferment at least once a day (F) and 12 who did not eat soy every day but who had a vegetarian diet were classified as vegetarian (V). The contingency table is presented in Table 5. No weight difference was recorded between groups (data not shown). A dot plot was drawn associating plasma levels to day-time. No significant correlation could be drawn from this plot indicating that the inter-individual variation is greater than the time course variation. This can also be due to the fact that women are used taking their Biopause treatment whenever they want: in the morning or in the evening.

3.6. Global analysis

When analysing the plasma levels three classes of values were constituted, a low, a median and a high. These classes were reported on a multiple correspondence analysis graph and it was observed that the lowest were plotted on the left, the median in the middle and the highest on the right. When plasma levels grouped

Table 3
Isoflavone in diet supplement exclusively based on soy and commercialised in France

Food supplements	Laboratory	Claimed doses/tablet	Genistein mg	Daidzein mg	Total mg/tablet	Prescription per day	Dose/day mg
Biopause	Monin Chanteaud	?	1.43	1.02	2.45	2	4.90
Bioptimum Soja	Boiron	?	3.11	8.97	12.08	2	24.16
Efodyne	Yves Ponroy	20 mg	1.49	6.69	8.18	1	8.18
Elugyne	Dolisos	?	2.66	10.64	13.30	1	13.30
Estronat	Lescuyer	40 mg	6.62	6.48	13.10	3	39.30
Evestrel	Theramex	37.5	11.61	9.19	20.80	2	41.60
Feminine	Medikem	?	3.27	1.46	4.73	2	9.46
Gydrelle	Iprad Dr Fagnen	45 mg	4.48	13.27	17.75	2	35.50
Gynalpha	CCD	76 mg	28.60	64.20	92.80	1	92.80
Gynalpha	CCD	38 mg	12.07	15.00	27.07	2	54.14
Gynosoya	Codifra	37.5 mg	27.00	7.36	34.36	2	68.72
Isoyam	Starvital	10 mg	1.69	1.45	3.14	3	9.42
Macasoyam	Fenioux	15 mg	2.22	4.85	7.07	6	42.42
Menolig	Vichy	20 mg	11.30	5.57	16.87	4	67.48
Oligoforme 50	IDO	?	0.43	0.17	0.60	4	2.40
Preluzelle	LPF	30 mg	8.65	10.75	19.40	2	38.80
Phytofemme (iso)	Superdiet	20 mg	4.89	3.97	8.86	2	17.72
Phytosoya	Arkopharma	17.5 mg	1.58	6.59	8.17	2	16.34
Sojacal	Novagyn	?	9.55	6.31	15.86	2	31.72
Sojamag	Novagyn	?	10.70	6.10	16.80	2	33.60
Sojyam	Tonipharm	?	5.91	2.80	8.71	3	26.13
Soyolig	Vichy	37.5 mg	11.86	6.66	18.52	2	37.04

according to the prescription were plotted on the graph, the group "prescription 0" appeared on the left with the lowest isoflavone plasma values, the group "prescription 2" appeared in the middle and the group "prescription 4" appeared on the right together with the highest plasma levels. Finally when plasma values analysed according to diet were plotted ferment and vegetarian groups appeared on the left with the lowest isoflavone values, the omnivorous groups appeared in the middle and soy eaters were plotted on the right with prescription 4 and the highest plasma values. Because the soy eating group was further on the right than was the prescription 4 group it could be said that the diet effect was stronger than the prescription on the isoflavone plasma levels.

3.7. Prescription effect

The data collected on isoflavone plasma levels in the prescription groups are presented in Table 6. These data show that the higher the prescription, the higher the mean isoflavone plasma content. The mean plasma levels are 46.6 ± 100.4, 146.31 ± 133.9 and 177.2 ± 104.8 ng/ml for 0, 2 and 4 tablets a day, respectively. The difference between the female controls and the treated ones is significant but the difference between 2 tablets per day (4.9 mg/day) and 4 tablets per day (9.8 mg/day) is not. For each group the standard deviation is quite considerable showing a great inter-individual difference, and the data distribution does not follow the normal law.

Table 4
Isoflavone intake corresponding to a "normal" consumption of French soy-food

Soy-food	Genistein	Daidzein	Total	Volume/day	Dose/day
Liquid diets based on soy milk					
Tonyu	91.37 μg/ml	49.57 μg/ml	140.94 μg/ml	250 ml	35.23 mg/day
Yoghurt	44.70 μg/ml	37.40 μg/ml	82.10 μg/ml	2×110 ml	18.06 mg/day
Chocolate soy milk	115 μg/ml	70 μg/ml	185 μg/ml	250 ml	46.25 mg/day
Vanilla dessert	120 μg/ml	60 μg/ml	180 μg/ml	2×110 ml	39.6 mg/day
Vanilla cream	110 μg/ml	65 μg/ml	175 μg/ml	2×110 ml	38.5 mg/day
Soy cream	70.08 μg/ml	63.16 μg/ml	133.24 μg/ml	10 ml	2.66 mg/day
Soy based infant formula μg/ml of reconstituted milk					
Modilac soja	17.40 μg/ml	13.10 μg/ml	30.50 μg/ml	900 ml	27.45 mg/day
Nutrilon soja	26.50 μg/ml	11.60 μg/ml	38.10 μg/ml	900 ml	34.29 mg/day
Gallia soja	22.20 μg/ml	11.20 μg/ml	33.40 μg/ml	900 ml	30.06 mg/day
Prosobee soja	11.15 μg/ml	6.35 μg/ml	17.50 μg/ml	900 ml	15.75 mg/day
Solid diets based on soy milk					
Herbs cheese no. 1	351.10 μg/g	300.90 μg/g	652 μg/g	2×25 g	32.6 mg/day
Herbs cheese no. 2	285.80 μg/g	185.15 μg/g	470.95 μg/g	2×25 g	23.54 mg/day
Solid diet based on soy protein					
Sausages	82.21 μg/g	40.64 μg/g	122.85 μg/g	2×45 g	11.06 mg/day
Smoked Tempeh	165.33 μg/g	112 μg/g	277.33 μg/g	50 g	13.87 mg/day
Tofu 1	150.33 μg/g	71.22 μg/g	221.55 μg/g	100 g	22.15 mg/day
Tofu 2	48.05 μg/g	46.16 μg/g	94.21 μg/g	100 g	9.42 mg/day
Powder for instant drink	67.29 μg/g	50.34 μg/g	117.63 μg/g	3×13 g	4.59 mg/day
Liquid diet based on soy					
Soy sauce	6.27 μg/ml	11.27 μg/ml	17.54 μg/ml	4 ml	70.16 μg/day
Soy lecithin (ng/g)					
Soy lecithin	179.15 ng/g	686.00 ng/g	865.5 ng/g	10 g	8.65 μg/day

Table 5
Contingency table of the groups identified in the women population followed in the survey[a]

Prescription	Diet				
	Ferment (F)	Omnivorous (O)	Soy-eater (S)	Vegetarian (V)	Total
0	12 (6.59%)	4 (2.19%)	0 (0%)	3 (1.64%)	19 (10.44%)
2	42 (23.07%)	56 (30.76%)	34 (18.68%)	6 (3.29%)	138 (75.83%)
4	4 (2.19%)	12 (6.59%)	6 (3.29%)	3 (1.64%)	25 (13.73%)
Total	58 (31.87%)	72 (39.57%)	40 (21.97%)	12 (6.59%)	182 (100%)

[a] Results are expressed as number of subjects in each group, in parentheses respective percentage are calculated.

3.8. Dietary effect

The data obtained from the isoflavone plasma levels in the different diet groups are presented in Table 7. Total isoflavone plasma level includes equol. It appears from these data that the lowest isoflavone plasma content is recorded in soy-free vegetarian women (89.67±127.9). Then comes the group of women taking ferments, with a mean plasma level of total isoflavone of 100.1±102.3 ng/ml. The omnivorous women presented

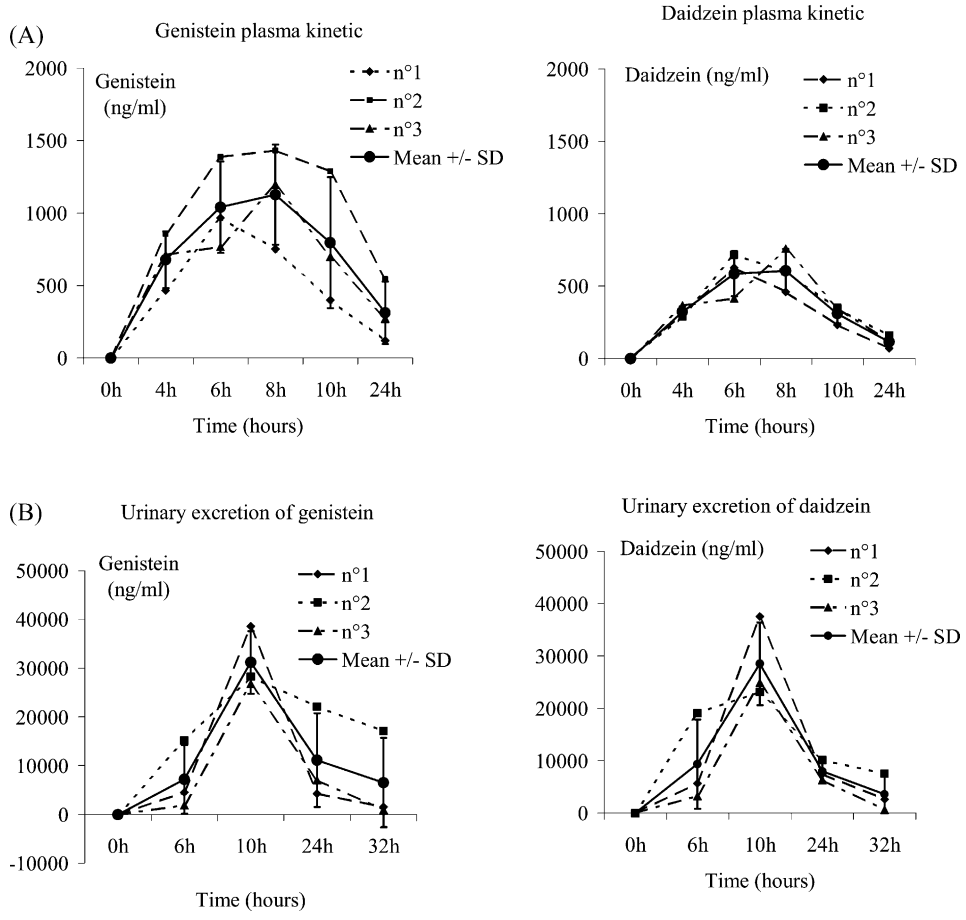

Fig. 2. Plasma (A) and urine (B) measurements in women after a single intake of 100 mg total isoflavones containing 2/3 genistein and 1/3 daidzein.

Table 6
Isoflavone plasma levels in women under 0, 2, or 4 tablets per day, i.e. 0, 4.9 or 9.8 mg of total isoflavone in equivalent aglycone

Prescription	0 tablet/day $n=19$	2 tablets/day $n=138$	4 tablets/day $n=25$
Total isoflavones ng/ml			
Mean plasma level±S.D.	46.6±100.4 (a)	146.31±133.9 (b)	177.2±104.8 (b)
Min. and max. plasma level values	0–400.8	0–697.5	0–439.3
Median	0	138.8	174.7
Genistein ng/ml			
Mean plasma level±S.D.	24.9±62.0 (a)	69.9±68 (b)	86.49±55.33 (b)
Min and max plasma level values	0–260.0	0–367.7	0–213.9
Median	0	60.7	76.78
Daidzein ng/ml			
Mean plasma level±S.D.	21.6±41.22 (a)	77.21±73.66 (b)	91.2±57.0 (b)
Min and max plasma level values	0–140.8	0–350.4	0–234.4
Median	0	69.9	89.33
Equol ng/ml			
Mean plasma level±S.D.	5.4±11.3 (a)	11.95±18.1 (a)	13.44±21.0 (a)
Min and max plasma level values	0–30.3	0–102.9	0–101.3
Median	0	0	0

Different letters indicates a significant difference ($P<0.05$) between the different treatments (ANOVA followed by post-hoc SNK test).

Table 7
Isoflavone plasma levels in the different diet categories

Diet habits	Ferments $n=58$	Vegetarian $n=12$	Omnivorous $n=72$	Soy eater $n=40$
Total isoflavones ng/ml				
Mean plasma level±S.D.	100.1±102.3 (a)	89.67±127.9 (a)	144.6±118.9 (a,b)	232.8±152.2 (b)
Min and max plasma level values	0–400.8	0–343	0–572.4	0–697.5
Median	109.4	0	152.4	191.6
Genistein ng/ml				
Mean plasma level±S.D.	46.5±51.84 (a)	43.7±66.2 (a)	68.7±59.1 (a)	113.2±81.3 (b)
Min and max plasma level values	0–260.0	0–183.2	0–252.3	0–367.2
Median	43.9	0	66.8	88.4
Daidzein ng/ml				
Mean plasma level±S.D.	53.2±55.6 (a)	47.0±63.7 (a)	77.2±67.2 (a,b)	118.3±83.4 (b)
Min and max plasma level values	0–254.6	0–159.8	0–320	0–350.5
Median	55.4	0	75.3	98.9
Equol ng/ml				
Mean plasma level±S.D.	8.9±11.6 (a)	8.0±15.4 (a)	11.7±19.1 (a)	14.8±20.7 (a)
Min and max plasma level values	0–36.7	0–47.1	0–102.9	0–101.3
Median	0	0	0	11.4

Different letters indicates a significant difference ($P<0.05$) between the different diets (ANOVA followed by post hoc SNK test).

total isoflavone plasma levels of 144.6±188.9 ng/ml and the soy-eaters 232.8±152.2 ng/ml. The difference is significant between the vegetarian women and the women taking ferment on one hand, and the soy-eaters on the other hand. The isoflavone concentrations in plasma from omnivorous women are not different from those measured in the other groups.

3.9. Equol production

Table 8, presents the percentage of equol producers in each group. It can be seen that the differences are never significant. Considering the global data, the rate of equol producers appears to be of 43±7%.

4. Discussion

4.1. Validation of the assay

All the data presented here were obtained using ELISA techniques for genistein, daidzein and equol described by Bennetau-Pelissero et al. (2000) and Le Houérou et al. (2000). The extraction procedures as well as the hydrolysis were validated using HPLC with DAD detector for foodstuffs and food-supplements and HPLC coupled to cool array detection for plasma and urine measurements (data not shown). Table 1, showing the cross-reaction tests, clearly indicates that these assays can only be used for soy or kudzu analysis or for fluids coming from people who consume these plants. Indeed, the cross-reaction of anti-genistein and anti-daidzein for biochanin A and formononetin, respectively do not allow measurements in alfalfa or clover or on fluids from subjects submitted to these plants. In Western diet, sources of biochanin A and formononetin exist (peas, chick peas, lentils, etc...) but as mentioned by Mazur (1998) the quantities of these isoflavones brought by these vegetables are about 1/100 of that brought by soy or kudzu. Moreover Setchell et al. (2001), demonstrated that when biochanin A and formononetin are given to humans the conversion into genistein and daidzein, respectively, by the gut microflora is very efficient in human as already demonstrated in other species. Therefore, although our antibodies exhibit significant cross-reactivities with biochanin A and formononetin and because dilutions are always performed before analysis it is considered that the ELISA technique specifically based on the antibodies described here is reliable. Moreover, all measurements were done at two different dilutions and the absence of unexpected cross reaction can be checked comparing the doses obtained at these two dilutions. In our case, the inter-dilution variation is always less than 15%. This is of the same order of magnitude as the inter-assay variation and confirms the absence of cross-reactions.

Table 8
Percentage of equol producers in each group

Types	% Of equol producer	Number of subject	Confidence interval
Diet			
Ferment	43.1	58	29.1–55.1
Vegetarian	33.3	12	9.9–65.1
Omnivorous	41.6	72	31.1–55.3
Soy-food	55.0	40	38.6–70.7
Prescription			
0 tablet	21.0	19	6.1–45.6
2 tablets	45.6	138	36.4–53.6
4 tablets	44.0	25	24.4–65.1

Table 2 obtained by transforming the initial data of the Acatris ring test from conjugated forms into aglycones, shows that our data (laboratory 9) are consistent with that of other laboratories. As previously mentioned, all the data from other laboratories were obtained using physico-chemical techniques after various extraction steps. Verbruggen and Rooijen (2001) mentioned large variations in the initial values coming from large differences in the proportions of each conjugated form. Indeed, it appeared that depending on the extraction procedure, the proportion between malonyl, acetyl, glycoside and aglycone forms could vary greatly. When all data were transformed into aglycone forms the discrepancies between values decreased. However, other problems were mentioned due to (1) the great variety of external standards used to calibrate the HPLC techniques (2) the great variety of internal standard used and the moment when it was added in the extraction process, which could influence the recovery percentage. Moreover, most of the time, spectra of those standards were read at a define wavelength without consideration of their proper extinction coefficient. Nevertheless, and although the true values are unknown, it seems that there is a consensus which appears between the same laboratories. For samples 1, 2, 3 and 4 the data obtained by ELISA fits quite well with the mean of all the data from all the laboratories. ELISA values are always a bit higher than the mean but they are in accordance with that of several consensual values. In all cases, data obtained by the ELISA technique are reproducible. Considering samples 5 and 6, the ELISA values are higher than the mean. Indeed, the mean is obtained from certain data that seams to be too low to be reliable but on the other hand ELISA data are higher than all other data at least for genistein. It is not due to cross-reaction with either biochanin A or formononetin since none of the other laboratories mentioned these compounds using physico-chemical techniques. This can be due to the great dilution having to be performed for the measurement of samples 5 and 6. This may have induced a problem when the data had to be corrected by the dilution factor (100,000 versus dilution 1000 for sample 1 and 2) to be expressed in μg/g. Therefore, ELISA, which is very sensitive cannot be used for very concentrated matrices unless extraction is performed on very small samples (0.01 g or even lower).

4.2. Isoflavone measurements in food-supplements

Measurements on food supplements lead to the same conclusions than those drawn by Setchell et al. (2001). Indeed, a great variability of isoflavone content in these supplements was noticed and discrepancies were sometimes found between the claimed doses and those effectively measured. Because no standardised assay method exists yet, the concentrations found in soy-based supplements have to be regarded with caution. Differences were found between batches from the same brand (data not shown) and the values presented in Table 3 are likely to represent a picture at a given time and may not reflect a constant situation. A discussion with a manufacturer also pointed out that in some cases the isoflavone content although mentioned on the packaging as isoflavones are in fact expressed in glycosides whereas we always measure data in equivalent aglycone. This explains why sometimes our measurements are lower than that mentioned on the packaging. When the conversion is done the results are close.

4.3. Isoflavone measurements in soy-food

For foodstuff analysis, the ELISA data can be compared to the work by Anderson and Wolf (1995) and for soy-based infant formulas to the several previous studies (Irvine, Fitzpatrick, & Alexander, 1998; MAAF, 1998; Setchell, Zimmer-Nechemias, Cai, & Heubi, 1997). Compared with data from Anderson and Wolf (1995), the present data are correct, being in the same range although generally lower. Because it has been shown that the isoflavone amount in soy-foods can vary from one trademark to another, and within the same brand, from one batch to another, the order of magnitude of concentrations is more interesting to consider than the values themselves. Considering soy-based infant formulas, the present data fit well with those of previous studies. Other authors found exposures between 16.2 and 47.0 mg/day for 4-month-old infants (Irvine et al., 1998; MAAF, 1998; Setchell et al., 1997) when the present data are comprised between 15.7 and 34.3 mg/day, also for 4-month-old babies. The Western diet is usually considered as including low amounts of phytoestrogens (Kleijn et al., 2001). However, the conjunction of health allegations about soy, together with the on going meat crisis (dioxin affairs, mad-cow disease...) has lead, in France, to an increase in soy-food consumption. If different soy food can be consumed each day by a person, intakes of 100 mg/day can be expected. We do not really know at the moment if such exposures can be beneficial or not. Anyway, it is likely to be highly dependant on physiological condition, age or sex.

4.4. Analysis of the kinetic study

Our results can be compared to those of Watanabe et al. (1998) and to those of Setchell et al. (2001). Watanabe et al. (1998) used 60 g of baked soy containing 50 mg of isoflavone (24 mg daidzein and 26 mg genistein). They obtained the plasma C_{max} 6 h after ingestion but did not collect any samples between 6 and 12 h after ingestion. The real peak, as found in our study, could then likely appear between 6 and 8 h after isoflavone

intake. Considering urinary data, it can be noticed that they found the C_{max} between 6 and 12 h. This corresponds pretty well to our findings. Watanabe et al. (1998) also mentioned that the daidzein urinary excretion was more important than that of genistein. We observe the same pattern, since, although daidzein only represents 1/3 of the total isoflavones ingested, its urinary C_{max} is nearly as high as that of genistein (28,752±7877 ng/ml versus 31,214±6432 ng/ml for daidzein and genistein, respectively). Looking at Setchell et al.'s work (2001), the comparison is not as easy since these authors used purified compounds whereas we used total soy extracts. The plasma C_{max} was then found 5.5 and 6 h after ingestion for genistein and daidzein in the aglycone form respectively. However, plasma C_{max} were found 9.3 and 9 h after ingestion for genistin and daidzin, respectively (β-glycoside forms of genistein and daidzein). In our experiment, soy extracts were given which may contain different types of conjugates. The urinary profiles were not followed in this study. Considering the concentrations, Setchell found C_{max} of 394±61 ng/ml after the ingestion of 50 mg of daidzein glycoside and 341±127 ng/ml after the ingestion of 50 mg of genistein glycoside. Our data are higher 1127±345 ng/ml for genistein and 607±149 ng/ml for daidzein. This difference can be explained by the simpler extraction procedure used, which avoided the loss of compounds provided that the isoflavone absorption by the gut is linear with increasing intake. Compared with that of Watanabe et al. (1998), however, our data could be relevant if the amount of isoflavone were expressed in equivalent aglycone in their study. Indeed, these authors found C_{max} of 660±175 ng/ml for genistein and 396±86 ng/ml for daidzein. The comparison is correct assuming that the absorption process varies linearly when exposure increases.

4.5. Plasma measurements in the menopausal women survey

The ELISA technique allows a great number of analyses in a short period of time. The plasma data collected during the Biopause® survey are consistent with previous plasma measurements in Western subjects. Most of the data yet available were obtained using physico-chemical techniques rather than immunological ones, except for two studies (Gooderham, Adlercreutz, Ojala, Wahala, & Holub, 1996; Uheara, Arai, Watanabe, & Adlercreutz, 2000). In Western subjects, Gooderham et al. (1996) chronically supplemented men with 69.54 mg isoflavones/day. Details of single or dual intake in the administration procedure were omitted as well as time lapse from the last intake to the blood sampling. Nevertheless, the authors found levels of 1404 nM of genistein plus daidzein (about 370 ng/ml). Franke, Custer, and Tanaka (1998) giving 37 mg of total isoflavones, found plasma levels of about 2000 nM, (520 ng/ml equivalent aglycones) after overnight fasting. Xu, Wang, Murphy, Cook, and Hendrich (1994) giving 44, 80 and 130 mg in one isolated intake to young American women, found isoflavone plasma levels of 1530 nM (400 ng/ml), 2290 nM (598 ng/ml) and 4390 nM (1146 ng/ml), 6.5 h after intake. Setchell et al. (2001), giving approximately 30 mg of genistein and daidzein in equivalent aglycone in a single intake, found C_{max} of 394±61 ng/ml (1551 nM) and of 1220±470 ng/ml (4520 nM) for daidzein and genistein, respectively, 6 h after ingestion. Finally, the ELISA data fit quite well with those from Franke et al. (1998) and Xu et al. (1994) although slightly higher than those of Gooderham et al. (1998) and lower than those of Setchell et al. (2001). This is still assuming that the gut absorption varies linearly with intake, which is not proven so far.

Considering the survey it appears that the higher the prescription, the higher the isoflavone plasma levels. Therefore, the assay is sufficiently sensitive and can be used for higher isoflavone exposures. However, inter-individual differences were great, because no specific diet was imposed upon the women participating in this study and because plasma sampling was done at different moments in the day as was treatment intake. The prescription "0" group functioned as a control since none of the women declared eating soy (Table 5). The isoflavone plasma level was not nil however, because five women among the 19 presented significant plasma concentrations of genistein and daidzein. This could be due to the intake of beans, peas, chickpeas, lentils, etc ... also known to contain phytoestrogens (Mazur, 1998).

Based on the isoflavone plasma levels in each group as well as on multiple correspondence analysis and on the statistical tests, the food effect is higher than the prescription effect. Although this is not a definitive proof, the amounts of isoflavone in soy-food are quite high. Calculated on the basis of "normal consumption", isoflavone intake from soy-food (Table 4) is higher than isoflavone intake based on Biopause® prescription (Table 3). Biopause® is one of the least concentrated soy-based supplements available on the French market (Table 3).

The dietary questionnaire was very simple so as to be voluntarily filled in by a great number of persons in only few minutes. Four groups of women were identified: omnivorous (O), vegetarian without significant soy intake (V), ferment-eaters (F) and soy-eaters (S). Low plasma levels recorded in V and F could be due to an acceleration of the intestinal transit, which could have reduced isoflavone absorption by the gut, mechanically. Indeed, manufacturers of ferment containing yoghurts advertise their products with this health allegation. The effect of bifido bacteria on the disappearance of daidzein and genistein was checked in vitro in our laboratory. These bacteria, incubated with soymilk, were

shown to have no influence on the amount of isoflavone (data not shown). This sustains a mechanical effect rather than a biochemical effect.

5. Conclusion

As a conclusion, a new technique is described and validated for phytoestrogen measurements. It is relevant for soy-foods and soy-based food-supplements as long as their concentrations in genistein and daidzein are not too high. If they are, the dilution process needed to fit the data into the standard curve can induce over-estimations. The ELISA technique is based on the use of specific antibodies directed against genistein, daidzein and equol. No other isoflavones can be measured by this technique. In addition, because of significant cross-reactions of anti-genistein with biochanin A and of anti-daidzein with formononetin, this technique is only relevant to assay soy or kudzu plants or food and for biological fluids from animals or humans exposed to these types of food. It cannot be used for clover or alfalfa exposure. Indeed, these are the only vegetables which can lead to high plasma levels of biochanin A, formononetin, genistein, daidzein and eventually equol after ingestion and gut flora activity, in humans. Nevertheless, this new technique sustains the comparison with other physico-chemical techniques in reliability and sensitivity as shown by the Acatris ring test. It is cheap, rapid, sensitive and does not require complex extraction steps or methods. It can be used for large-scale studies, whether surveys or epidemiological studies. It was used in this study for a survey on 207 women under a low isoflavone supplementation and was shown to discriminate between treatments.

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