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Traditional and Domestic Cooking Dramatically Reduce Estrogenic Isoflavones in Soy-Foods

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Abstract: Soybean a pulse of considerable nutritional value due to its high protein, fibers and polyunsaturated fatty acid (PUFA) contents, also contains phytoestrogenic compounds that definitely hinder its recommendation for general consumption. Contrarily to ancient times when soybeans were boiled, modern commercial soy-foods can contain up to 150 mg/100g of estrogenic isoflavones. Interestingly, current estimations of isoflavone intake in the literature do not distinguish between the origins of soy-food i.e. home-made or commercial. As a result, the isoflavone exposure in Asian countries may well be over-estimated. This study aims to demonstrate, based on step-by-step monitoring of isoflavones, that traditional and domestic treatments, leveraging isoflavones water-solubility, can indeed significantly reduce their content in soy-foods. Indeed, when compared to commercial foods, the isoflavone contents was found to be 20, 2.6, 4.5 and 9.8 times lower in "home-made" soy-juice, tofu, tempeh and miso, respectively. Additionally, water soaking was found to reduce the isoflavones levels in soy-textured-proteins by more than 70%. Hence, this simple process has the potential to help drastically reduce overall xenoestrogens exposure. This study could serve as a basis for establishing the isoflavones Reference Dose and issuing food safety guidelines.

Keywords: soy-food; phytoestrogens; genistein; daidzein; cooking practices; water treatments; human exposure; health

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1. Introduction

Soybean is recognized as the foodstuff with the highest concentration of isoflavones (IFs), which exhibit estrogenic activities. Compared to other xenoestrogens, IFs bear the closest resemblance to estradiol and are highly concentrated in food sources [1,2]. The effects of IFs can vary from beneficial to adverse, contingent on the physiological status of the consumer and the ingested quantity [3]. In 2008, the National Toxicology Program (NTP) of the USA classified genistein (GEN), the primary soy-isoflavone, as reprotoxic and carcinotoxic in rats at a dosage of 35 mg/kg/day [4,5]. Given the absence of a reliable NOAEL (No Observable Adverse Effect Level), Food Safety Agencies should propose a common Reference Dose for the estrogenic IFs that should not be exceeded. This limit should also consider the actual exposure in Asian countries. Estimating this exposure necessitates reliable measurements in food-stuffs, an issue this study aims to address.

Due to potential adverse effects in certain population categories, it may be beneficial to reduce the levels of estrogenic IFs in the diet intended for the general population, reserving IFs for specific applications and circumstances. This cautionary approach is often disputed, with arguments that soy is traditional in Asia and that no adverse effects, and even beneficial ones, have been reported in the past [6]. However, does high soy consumption necessarily equate to high IFs exposure?

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A close examination of soy's history in China reveals that written testimonies were frequently lost and reconstructed over centuries, making it challenging to trace. The first reliable reports date from the Han dynasty (206-BCE to 220 CE). They described agricultural practices that could be dated back 2000 years before reporting, making it difficult to distinguish factual information from myths [7]. Although, soy has been cultivated for ages, the destruction of written sources prevents the confirmation of a time period for soybean use as a foodstuff. For instance, it was primarily used as a green manure before the Han dynasty, when the major crops cultivated were millet, wheat, rice, hemp, mung beans, and sesame [8]. In fact, soybean can be cultivated even on low-quality soils due to its ability to fix nitrogen in soils, enhancing subsequent cultures. As such, it was used for over 1000 years in quite complex crop rotations [9]. Soy was easily stored for up to three years since it contained many antinutritional factors which reduced pest (rodents or birds) attacks in the fields or during seed storage. Initially, it was mainly used to feed animals and was considered a starvation and war crop around 100 BCE [7,10]. However, soy was not highly appreciated due to its poor taste and the flatulencies and the stomach pains it induced. The most effective method to improve soybeans' digestibility, at that time was to boil them. Early fermented soy foods were seemingly reported 1200 BCE, in Korea. Soy was then considered a potential food for human beings [9]. In China, according to Baraibar-Norberg and Deutsh [7], *shi* (fermented soybeans) was found in Han tombs dated from 200-150 BCE. Fermentation which allowed better conservation and enhanced taste, required the use of clean and sterile beans before the development of specific molds, implying water boiling [9]. Soy curd like tofu seemed to appear during the late Han dynasty. Again, the traditional recipes included several cleanings and rinses as well as prolonged water boiling [11].

Such treatments applied to beans or dehulled beans have been reported to yield the inactivation of antinutritional factors and the removal of estrogenic IFs while preserving proteins [12], fibers, and polyunsaturated fatty acids (PUFAs) among ω3 and ω6 families [13]. In contrast, modern industrial processes primarily involve physical constraint treatments such as pressing and extrusion, which may lead to some concentration of IFs. Therefore, when dietary reports in the literature, suggest that Asian populations are exposed to high levels of IFs, based solely on high soy-food consumption, it appears to be a questionable assumption if the food origin is not specified.

Additionally, when data associating estimated IFs intakes and IFs in body fluids are gathered, a discrepancy emerges between Western and Asian populations. The same estimated exposure leads to 1 to 1.5 µM in Western people' blood, while it is about 0.2 to 0.3 µM for Asians. This was previously questioned by Vergne et al., in [14] when the pharmacokinetics of GEN and Daidzein (DAI) were assessed concurrently in French and Chinese students. The study revealed only a small difference in the C_{max} of DAI during chronic soybean challenge and it was hypothesized that, in Asian students, the gut flora may negatively influence the IFs bioavailability. However, this finding alone could not explain the significant difference observed in Asians and Westerners' body fluids concentrations of IFs. Thus, the hypothesis of a misestimation of IFs intakes remained.

This study was designed to address this issue. Its objective is to demonstrate that traditional recipes, and by extension the domestic ones inspired by them, reduce significantly IFs levels in Asian soy-foodstuffs. Hence, GEN and DAI concentrations were measured on soy-foods prepared under domestic conditions by ELISA. As the exact step for potential IFs removal was unknown, the processes were analyzed step by step to determine their impacts on the concentration of these two major estrogenic IFs. The results should indicate that traditional exposure to IFs in Asia is not as high as suggested by recent data [15,16] which established the IFs exposure at a median of 13.5 mg/day in China and at 21.4 mg/day in Japan.

The foodstuffs selected were tempeh and miso for fermented food and soy-juice and tofu for unfermented food, these being among the most common traditional dishes. Soy textured proteins were also treated under domestic conditions since they can be used by Western consumers. A comparison was then made with commercial industrial equivalent products analyzed here and in a previous study by the same assay technique. The health effects of estrogenic IFs are then discussed as well as the most probable consequences of this finding on the estimation of Asians' IFs exposure.

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2. Materials and Methods

2.1. Materials

All soy-matters *i.e.* whole beans, dehulled beans and textured proteins were supplied by the organic producer of soy proteins Biopress®/Berkem® (Tonneins, France). Dehulled beans were sifted before use to remove soybean dust. Textured soy proteins were sorted in four different particle-size groups: large (12-20 mm), medium (8-12 mm), fine (5-8 mm) and extra-fine (2.5-5 mm). Rice-koji ferments *i.e. Rhizopus oligosporus* for tempeh and *Aspergillus flavus* var *orizae* for miso as well as miso starter were bought from a local organic store. The commercial products plain tofu and tempeh analyzed as a basis for comparison were purchased from French supermarkets.

The chemical reagents were acquired from MERK (Fontenay-sous-bois, France) and Sigma-Aldrich (Saint Quentin Fallavier, France), unless otherwise stated. The samples and reagents aqueous solutions were made in ultrapure water obtained from an Elga Veolia® (High Wycombe, UK) instrument.

2.2. Methods

2.2.1. Traditional Recipes

2.2.1.1. Soy Juice

Soy juices were prepared from either 100 g of dehulled beans or whole beans. The first soaking step lasted 12 hours and during which water was renewed 0, 1, 2, 3 or 4 times. Then, the beans were mashed in 700 mL of either soaking water or clean water. The different mixtures obtained were individually filtered on a piece of fabric and the corresponding okara kept aside. For each type of juice collected, 350 mL was diluted twice with water to reach the commercial concentration of soy-milk. After a boiling step to reduce all anti-nutritional factors, it was allowed to cool down to room temperature before storage at 4°C or -32°C. At each stage of the process, 3 samples were collected and analyzed individually.

2.2.1.2. Tofu

Tofu samples were prepared from the different soy-juices obtained just before the last dilution step as previously described. After boiling, nigari ($MgCl_2$) was added at a final concentration of 5g/L and the juice was left to curdle for 30 min. The curds were poured onto a tofu press and the whey discarded by pressing for 30 minutes at 25 500 Pa.

2.2.1.3. Tempeh

The recipe is based on a traditional process described by Fernandez-Lopez *et al.* [17]. Dehulled beans were rinsed 3 times with tap water before being cooked in boiling water for 20 min and then left soaking in cooking water for an extra 20 min. During this first cooking step the foam formed on top of water was removed. These precooked beans in turn underwent the same 3 processing stages as the dehulled beans. After the second cooking, beans were lightly dried spread on a baking sheet in an oven at 60°C for 10 min. Finally, the beans were mixed with white vinegar and *Rhizopus oligosporus* ferment, reaching concentrations of 6 mL/100g and 500mg/100g, respectively. The mixture was poured in zip-lock plastic bags, hole-pierced with a fork, and left in a heat chamber at 28°C for 72h.

2.2.1.4. Miso

The traditional recipe of miso was taken from The Japanese Lab [18] which describes how to make light color miso. 100g of dehulled beans were cooked in boiling water for 3 hours during which the boiling water was renewed twice. They were cooled-down to room temperature and mixed-up with the equivalent amount of rice-koji, *i.e.* rice pre-incubated with *Aspergillus flavus* var *oryzae* and 133 mg of miso-starter. The obtained mixture was round into balls which were deposited and pressed

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into a large-neck glass-jar to avoid air pockets. Its surface was sprinkled with salt and then covered by a food-grade plastic film supporting a ballast. The jar was closed and the mixture was left to ferment for 8 months.

2.2.1.5. Hydrated Soy Proteins

Four sizes of textured soy-proteins were tested. In each case, 100g of matter were soaked in 1 L of tap water to hydrate for 30 minutes during which 0, 1, 2 or 3 water renewals were done. In the case of 1 renewal, it occurred after 30 minutes; for 2 renewals, they occurred after 15 and 30 minutes; for 3 renewals, they occurred every 10 minutes. Two water temperatures were tested: 22°C and 65°C.

2.2.1.6. Commercial Tofu and Tempeh

A consumer portion, either 100g of tempeh or 125g of tofu, was immersed in 1 L of deionized water for a total of 15 minutes, with two water changes at 5 and 10 minutes before which the foodstuff was dried on absorbent paper and weighted. Water was gently stirred for 1 min at each water renewal. Samples were collected at 0, 5, 10 and 15 minutes of soaking. IFs, i.e. GEN and DAI in aglycone forms, were assayed at each time set.

2.2.2. ELISA

2.2.2.1. Samples Preparation

To perform the IFs analyses by ELISA, the samples were first subjected to an extraction as described below. Thereby, 1 g of mortar-crushed soy-based material was dispersed into 50 mL of water by a two-step stirring, at room temperature for 20 min and then at 100°C for 10 min. After cooling down to room temperature, 500 µL samples were collected in triplicate under stirring to insure homogeneity. Two milliliters of acetate buffer (sodium acetate 0.1 M; EDTA 0.14 M; 100 UI.mL^{-1} penicillin G (Sigma, P-3032); 0.1 mg.mL^{-1} streptomycin (Sigma, S-6501)) at pH 5 were added to each sample vial together with 10 µL of β-glucuronidase aryl-sulfatase from *Helix pomatia* (Roche®, 10127698001, Mannheim, Germany) to allow the digestion of glycosylated IFs. The samples were then incubated overnight at 37°C under shaking [19]. Afterwards, the extraction of aglycone compounds was performed: 3 mL of acidified ethyl-acetate (500 µL HCl 38% per L) were added, the vial vortexed for 30 s, centrifuged at 500 g (Jouan™ CR3, Fisher Scientific, Illkirch, France) for 10 min at 4°C and finally stored at -22°C to allow phase separation. The organic phase containing the IFs in aglycone-forms was evaporated to dryness using a Speed-Vac (Thermo-Electron™ Corporation, Fisher Scientific, Bordeaux, France). Then, each sample was diluted in 0.5 mL of assay buffer, i.e. phosphate buffer saline (PBS) 0.01 M, 0.9% NaCl, 0.2% Tween, 1% DMSO, pH 7.3, and sonicated when required. Samples were stored at -32°C until processed for IFs analysis.

To assess the digestion and extraction recovery, the hydrolysis by β-glucuronidase aryl-sulfatase was monitored using genistin (EXTRASYNTHESE™, 1325 S, Genay, France), a pure compound used as a control reagent in an external standard run in parallel to each measured sample. The compound was dissolved at 1 mg.mL^{-1} in DMSO as the stock solution. In these operating conditions, the hydrolysis performance was always between 87 and 103%. In addition, a sample of chocolate soy-milk was run similarly to the tested samples. It was considered as the plate control. Recoveries greater than 100% can be explained by the high accuracy of ELISA measurements and inter- and intra-assay variations.

2.2.1.2. Assay

GEN and DAI were assayed in soy-matter and treatment-water using ELISA specific to each molecule, as explained by Shinkaruk et al. [20]. The primary antibodies were selected and obtained in previous works [21,22]. All glass-wares were coated with silicone (Sigmacoat®, Ref SL2) and all IFs solutions were handled with low retention tips.

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2.2.1.3. Characteristics of the ELISA

The soy-matter samples were diluted to 1/50 (v/v) for enzymatic digestion. The sensitivities of the GEN and DAI assays in food-matter were 10 µg.mg^{-1} and 6.5 µg.mg^{-1}, respectively. The intra-assay variation was never over 7% and the inter-assay variation obtained on different microtitration plates was always below 17% [20,21]. Assays were not considered if the "r" coefficient of the sigmoid calibration curve was < 98.5%. The final dilutions in the microtitration plates ranged from 1/50 to 1/800. All values are given here in aglycone equivalent.

2.3. Statistical Treatments

All data correspond to a mean value and its standard error of the mean (SEM)derived from at least triplicate measurements performed on three different microtitration plates. The significance was obtained by assessing the ν value according to the non-parametric Mann-Whitney test.

3. Results

Here are presented the IFs contents of tempeh and miso prepared according to traditional recipes. Data showing the impacts of water renewal during beans soaking on IFs contents for soy-juice and tofu domestic preparations are given next. The impacts of water rinsing on IFs contents of textured proteins of different sizes are also shown. Finally, the results of rinsing tests of commercial tofu and tempeh are presented. IFs levels in the okara samples collected during soy-juices and tofu makings are in supplementary Figure S1.

The concentrations of IFs were compared to those measured in commercial products bought in French supermarkets specifically for this study and also to those measured by our team for the same products using the same technique and published in [1]. The reference-data issued from [1] are also presented in Table 1.

Table 1. Genistein and daidzein amounts per portion of different types of soy food collected on the French market.

Type of product	Number of Measurements	Portion Size (g)	Mean GEN + DAI/portion (mg)	Standard Error of Mean/portion	Range of measurements*
Raw soy beans	5	100	84.6	8.3	72.5 to 100.6
Toasted soy beans	5	100	134.0	37.9	61.9 to 247.7
Edamame	2	120	49.2	14.1	35.1 to 63.3
Soybean flour	2	100	38.5	5.5	32.9 to 43.9
Protein isolate (sports)	3	165	52.2	12.3	39.8 to 64.5
Soy-based drinks	9	100	9.9	1.7	2.8 to 17.7
Soy-based desert cream	11	150	12.3	2.8	2.5 to 33.4
Soy-based yogurt	14	125	15.9	3.8	5.1 to 31.5
Soy-based cream	12	50 mL	4.5	5.9	3.1 to 6.7
Soy-based Vegan steak	19	100	27.8	3.5	4.1 to 51.2
Soy sausages	4	90	24.8	7.3	11.1 to 44.1
Soy-based raw pasta	2	100	23.7	1.6	22.1 to 25.3
Soy-based cooked pasta	2	100	8.75	0.6	8.2 to 9.3
Soy-based infant formulas	6	4 months infants	25.2	2.9	15.7 to 34.3
Tempeh	4	100	27.8	4.5	15.5 to 34.3
Tofu	13	100	34.8	5.9	9.4 to 79.8
Soy-based cheese	8	50	23.8	7.4	2.9 to 36.2
Miso	2	100	14.7	0.98	13.7 to 15.6
Soy sauce	9	10 mL	0.16	0.004	0.16 to 0.17

* Figures calculated from [1] and isoflavones are genistein + daidzein concentrations in aglycone forms.

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3.1. Impact of Traditional Process on Isoflavones Content in Tempeh and Miso

IFs levels measured at different stages of the traditional recipes of tempeh and miso are presented in Figure 1.

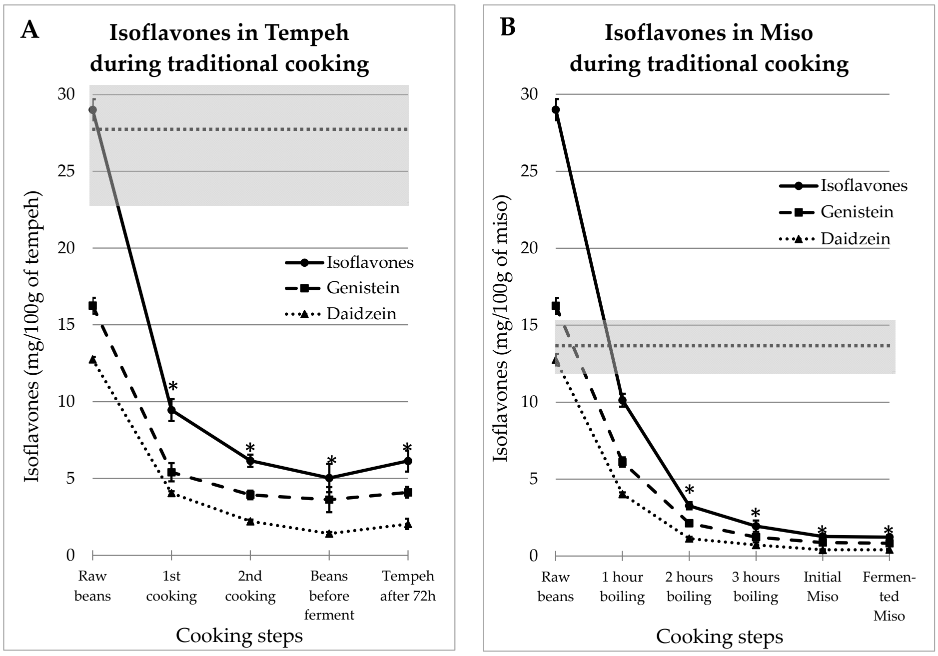

Figure 1. Evolutions of Genistein and Daidzein concentrations during the traditional preparations of tempeh (1A) and miso (1B). The grey zone covers the mean and SEM of the equivalent commercial products presented in Table 1. The stars indicate IFs values in food that were significantly different from the mean ± SEM of IFs content in commercial products (P<0.05).

As mentioned above, tempeh and miso were both made from dehulled beans which contained 16.5 ± 0.52 mg GEN/100g and 12.8 ± 0.17 mg DAI/100g. The IFs concentrations measured at the different preparation steps decrease in an asymptotic way as shown in Figure 1A for tempeh and Figure 1B for miso. The grey zones on the figure cover the range of values observed in industrial-equivalent food products. The stars indicate IFs values in prepared food that were significantly different from the mean ± SEM of IFs contents in commercial products (P<0.05). It can be seen that in both cases the resulting IFs levels are consistently much lower in “homemade” food than in commercial products. Moreover, this effect is the most important as soon as the first preparation step is done, additional steps bringing only slight reductions.

3.2. Impact of Water Renewals during Beans Soaking for Soy Juice and Tofu

3.2.1. Process Tested on Whole Soy-Beans

IFs were assayed in soy-juice and tofu prepared from whole beans, containing 32.8 ± 0.9 mg GEN/100g and 27.6 ± 0.12 mg DAI/100g, soaked for 12 hours with 0, 1, 2 or 3 water renewals. It should be noted that the 2 first soaking water batches contained dust and lipids but the subsequent water batches were clear. The concentrations of aglycone IFs in mg/100 mL of soy-juice and in mg/100g of tofu are given in Figure 2. It is seen that the amounts of IFs are always significantly lower (P<0.05) than those measured in commercial equivalent products. After the fourth rinsing, the amount of IFs was reduced by 43% in the juice and by 33% in the tofu sample. Indeed, the envelope of the seed prevents IFs from leaking in the water during the soaking steps.

The IFs levels measured in the okara produced during soy juice and tofu makings are shown in supplementary Figure S1. It can be seen that IFs decrease asymptotically. When okara was obtained from whole beans, 40% of IFs were removed after 3 water renewals, while almost 70% were eliminated after 4 water renewals on dehulled beans. The IFs decrease was significant after the first water renewal.

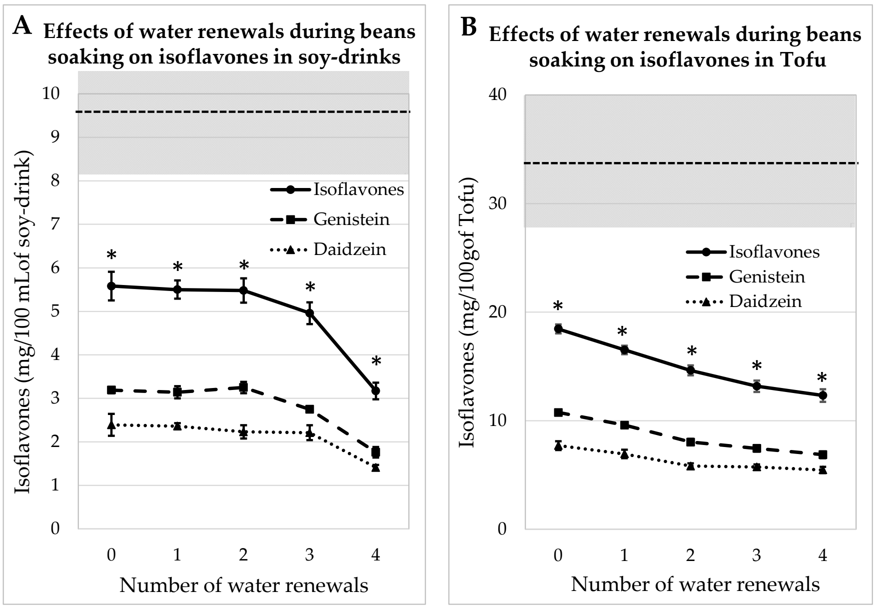

Figure 2. Evolutions of Genistein and Daidzein concentrations in soy juice (1A) and in tofu (1B) prepared from whole soybeans soaked for 12 hours in water renewed several times (1 to 4) or not renewed (0). The grey zone covers the mean and SEM of the equivalent commercial products presented in Table 1. * means a significant difference from commercial products (P<0.05).

3.2.2. Process Tested on Dehulled Beans

According to Traditional Tofu making experience in Debao Guangxi [11], it appears that the traditional recipe of tofu was based on dehulled beans. Therefore, the same soaking process as already described for whole beans was tested on dehulled beans. The concentrations of IFs i.e. GEN + DAI measured in each case are given in Figure 3. As observed in the precedent case, the "homemade" juice and tofu always contain significantly (P<0.05) less IFs than commercial equivalent products. When the number of water renewals increases, the IFs levels decrease in a quasi-asymptotic way.

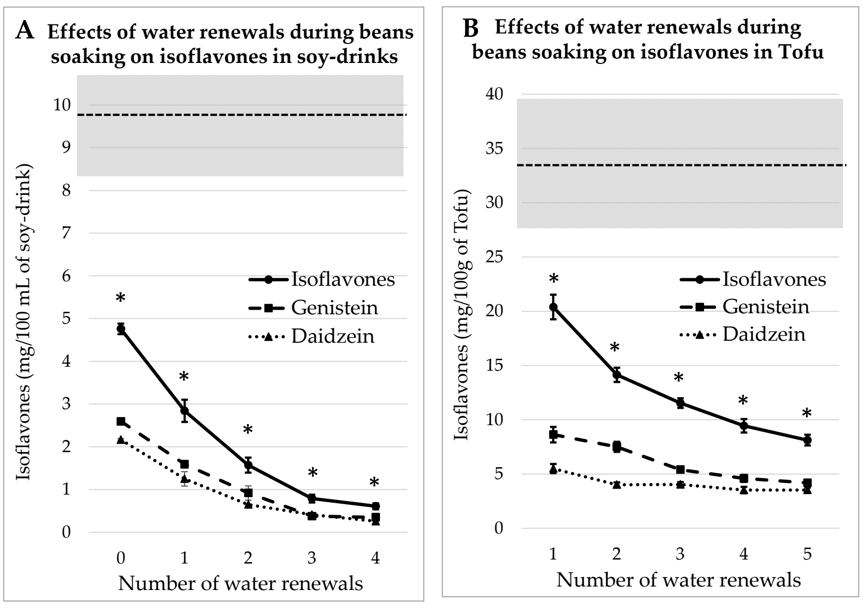

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Figure 3. Evolutions of Genistein and Daidzein concentrations in soy juice (1A) and in tofu (1B) prepared from dehulled soybeans soaked for 12 hours in water renewed 1 to 4 times or not (0). The grey zone covers the mean and SEM of the equivalent commercial products presented in Table 1. * means a significant difference from commercial products (P<0.05).

It can also be inferred by comparison between Figure 3 and Figure 2 that the additional water renewals are more efficient in removing IFs when applied on dehulled beans than on whole beans. After four rinses, the IFs concentration in soy juice is reduced by 87%, while in tofu it was reduced by 60%. The reason is probably that the lack of a seed coat eases the IFs leak into the water.

3.3. Impact of Water Renewals on the Levels of Isoflavones in Textured Proteins

The textured soy-proteins tested contained higher IFs concentrations than whole or dehulled soy-beans. Indeed, although the different products did not come from the same soy-matter, regular analysis of soy beans gave GEN + DAI concentrations of IFs aglycones around 60-70 mg/100g. In parallel, textured proteins were regularly measured at concentrations ranging from 90 to 110 mg/100g. This can be explained since the matter used did not come from the same batches of beans. In addition, textured soy-proteins were obtained from soy defatted cakes resulting from soy-seeds pressed for oil. As the extrusion process used does not significantly alter the IFs content and since oil represents about 20% in mass of the initial seeds, therefore the resulting textured soy-proteins are enriched in IFs.

3.3.1. Treatment with Water at 22°C

The concentrations of IFs in textured proteins hydrated for 30 minutes at room temperature with different rinsing steps are presented in Figure 4A. Here, the initial matter contained: 73.4 ± 3.8 mg GEN/100g and 35.2 ± 2.3 mg DAI/100g; 71.5 ± 2.4 mg GEN/100g and 35.1 ± 3.6 mg DAI/100g; 70.4 ± 2.4 mg GEN/100g and 38.3 ± 3.5 mg DAI/100g; 70.7 ± 0.8 mg GEN/100g and 36.5 ± 1.7 mg DAI/100g, for large, medium, fine and extra-fine particles, respectively.

From Figure 4A it can be seen that in all cases the first rinsing already results in the greatest drop in IF levels (P<0.05). After the third rinsing, the concentrations of IFs are decreased by 70.6%, 69.9%; 70.3% and 77.4% for large, medium, fine and extra-fine proteins, respectively.

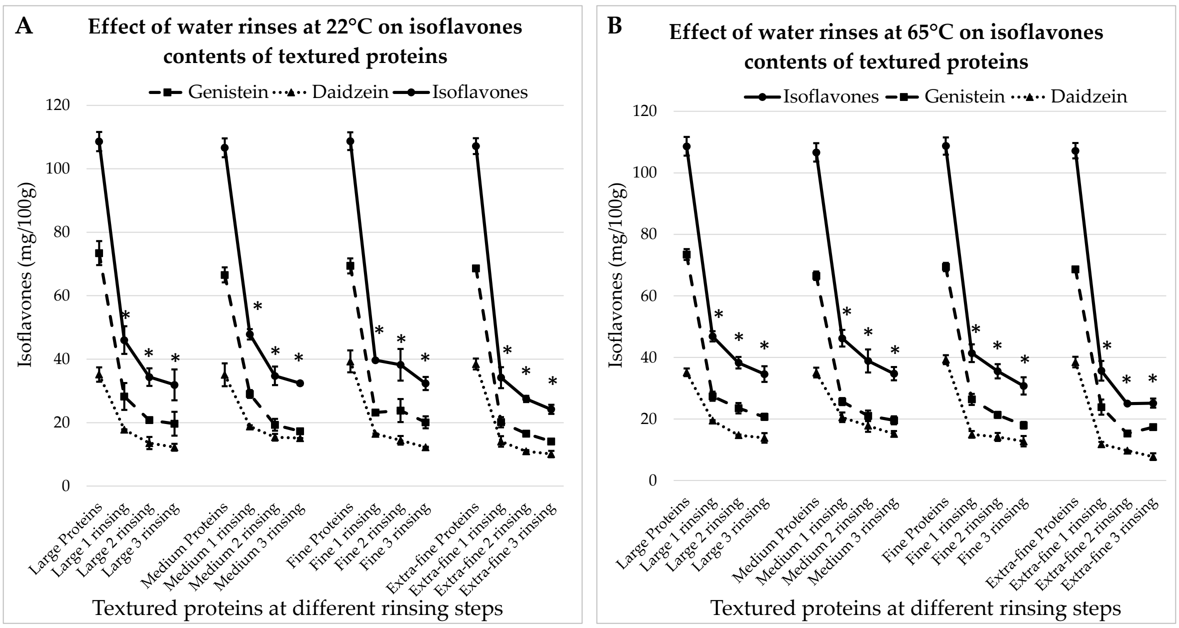

Figure 4. Effects of water rinses performed at 22°C (A) and 65°C (B) on the isoflavone contents of textured soy-proteins of different sizes, from large to extra-fine. * means a significant difference from initial IFs level (P<0.05).

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3.3.2. Treatment with Water at 65°C

The results for the treatment with hot water are presented in Figure 4B. They are very similar to the previous case: after the first rinsing, the concentrations of IFs decrease significantly (P<0.05); after the third rinsing, they decreased by 68.1%, 67.4%, 71.7% and 76.5% for large, medium, fine and extra-fine particles, respectively. Hence, IFs removal efficiency is not significantly influenced by the soaking temperature. However, for both temperatures the difference in the results is significant between the large and extra-fine proteins. At room temperature, after 3 rinsing steps, the total IFs contents are 31.9 ± 4.9 mg/100g and 24.2 ± 1.5 mg/100g in large and extra-fine textured proteins, respectively. At 65°C, the results are 34.6 ± 2.6 mg/100g and 25.1 ± 1.5 mg/100g in large and extra-fine textured proteins, respectively.

3.4. Impact of Domestic Rinsing on Isoflavones Content of Comercial Tofu and Tempeh

A consumer may prefer to buy ready-to-use tofu or tempeh and of course in that case the levels of IFs can be quite high as shown in Table 1. The commercial products used here for the domestic rinsing test, namely plain tofu contained 16.4 ± 0.92 mg GEN/100g and 11.6 ± 1.0 mg DAI/100g and plain tempeh contained 19.3 ± 0.18 mg GEN/100g and 17.2 ± 0.44 mg DAI/100g. The IFs levels corresponding to the different rinsing steps are presented in Figure 5.

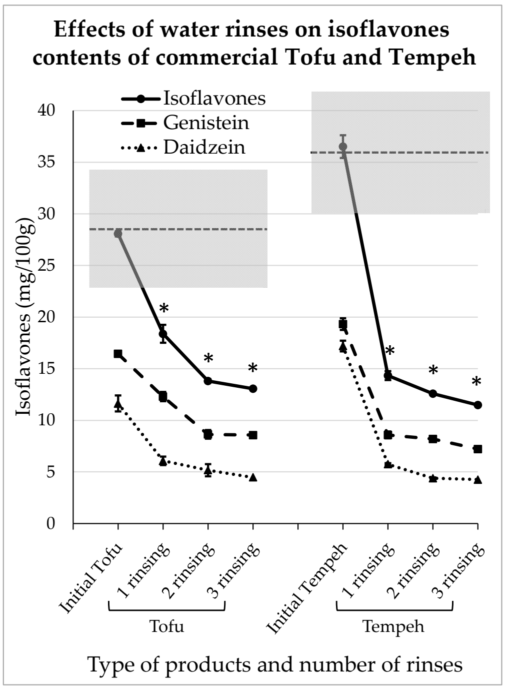

Figure 5. Reductions of isoflavones in commercial tofu and tempeh obtained after domestic water rinses. The stars indicate a significant difference in isoflavones concentration compared to the initial product (P<0.05).

Rinsing with water seems effective on commercial products provided that their texture is strong enough to withstand such treatment. In this experiment, the products were not significantly impregnated with water as they conserved their firmness and weight before and after each rinsing step. However, as can be seen in Figure 5, just after 10 min of contact with water the IFs levels are significantly decreased (P<0.05). Indeed, in tofu they are reduced at 12.3 ± 0.17 mg and 6.1 ± 0.7 mg for GEN/100g and DAI/100g, respectively. After 20 minutes of contact and 2 water renewals, they were reduced to 8,57 ± 0.41 mg and 4.5 ± 0.6 mg of GEN/100g and DAI/100g, respectively. Similarly, in tempeh after 10 min of rinsing the IFs concentrations were reduced to 8.6 ± 0.6 mg and 5.7 ± 0.5 mg for GEN/100g and DAI/100g, respectively. At the end of the rinsing process, the amounts of IFs were reduced to 7.2 ± 0.03 mg and 4.3 ± 0.1 mg for GEN/100g and DAI/100g, respectively. As observed

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previously, the IFs removal followed an asymptotic scheme and the third rinsing step does not seem to be very efficient.

4. Discussion

Although raw soybean is the pulse with the highest content of proteins, it also contains phytic acid and tannins which reduce mineral absorptions, oligosaccharides which ferment in the gut giving flatulencies, lipoxygenases which decrease PUFAs quality, and saponins and sapogenins which degrade the lipid membranes of enterocytes [23]. Soybeans also contain hemagglutinins, which can induce the coagulation of white and red blood cells if they enter the blood stream, and protease inhibitors, the Bowman-Birk and Kunitz factors which reduce the proteins digestibility [23]. Finally, they bear also allergens and IFs [23]. This is why Baraibar Norberg and Deutsh [7] wrote that "*The same chemistry that protects the soybean against pests makes it partially indigestible for humans and conducive to flatulence and stomach pain*". However, barely heating soybeans considerably reduces all anti-nutritional factors except IFs which are thermoresistant. Fortunately, since IFs are in glyco-conjugated forms they are soluble in water and may be reduced via water rinsing. This effect was tested in different contexts in this work.

4.1. Data Analysis

As seen in Table 1, industrial soy-food currently found in supermarkets may contain quite high, although variable, amounts of IFs. Unfortunately, only very few have a mention of their IFs contents on the packaging. In France the Food Safety Agency advised in 2005 to limit IFs intake to 1 $mg.kg^{-1}day^{-1}$ [24]. As will be seen later, this "Reference Dose" should most probably be lowered nowadays due to toxicological results subsequently obtained by the USA NTP. The French Agency's dose represents less than a portion of toasted soybeans for an adult of 60 kg.

The experiments performed here, show that IFs can easily be removed from soy matter by simple water treatments. This effect was already shown by several authors [25]. By implementing traditional recipes of tempeh and miso, it was possible in this work to obtain foodstuffs containing almost 5 to 10 times less IFs than commercial equivalent products. This suggests that the ancient soy-consumption, traditionally in Asia, only brought small amounts of estrogenic IFs. This work also shows that simple water renewals during domestic beans soaking can dramatically reduce estrogenic IFs from subsequent preparations of soy-juice or tofu. It appears that treating dehulled beans is more efficient for IFs removal than treating whole beans as already observed [12]. Similarly, when water rinsing was tested on textured proteins of different sizes, IFs were more efficiently removed from fine or extra-fine proteins than from large or medium ones. The difference of IFs levels between large and extra-fine proteins after water treatment was significant ($P<0.05$). Moreover, using hot water does not bring additional efficiency to the process. These results are consistent with those previously published by Bensaada *et al.* [12].

Regarding proteins and lipids contents of soy products, they are not significantly reduced after water treatment (data not shown) [12]. Indeed, working on whole beans or dehulled beans allows the soy-cells integrity preservation. So losses of large molecules are limited while small molecules such as IFs or vitamins and minerals can leak into the water when they are not adsorbed to larger biological cell constituents. In this study, vitamins and minerals were not analyzed based on the assumption that this issue should not constitute a major matter of concern since this was already the case when soybeans were boiled and if soy is a part of a diversified diet.

Finally, it may be difficult to perform these treatments at an industrial scale and this is why the rinsing processes were also tested on commercial products and they proved to be efficient. In anyways, such water rinsing or soaking should be encouraged at domestic levels for most consumers who do not require estrogenic IFs intake. Food companies may also innovate, creating food products that could be easily treated at home.

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4.2. Traditional Treatments

The ancient texts mentioning soybean and the actual soy-cooking practices which derive from ancient traditions, both involve cooking steps in water. Such process was applied to reduce anti-nutritional factors including IFs. This occurred when water was discarded. Soybean washing, soaking and boiling were mandatory since the early times of soy-food history [7]. Boiling allowed to cook soybeans and to sterilize them before fermentation [9].

Considered as a low value crop, soybean was initially used for animal feeding. However, when under the Han dynasty there was an increase of the Chinese population livestock farming declined and land was increasingly used to produce plant foods for humans. During this period, veganism developed in Asia and foods of animal origin were essentially based on fish, poultries and pork. During the territorial expansion wars of the Han period, soy became a war food being easily stored and transported. Because of its antinutritional factors soy was not appreciated by human consumers and was always boiled. According to Lee *et al*. [9] fermentation was developed quite early to improve soy taste and preservation and most probably to improve nutritional quality of boiled soybeans. Indeed, the first remains of fermented soy were found with the first earth-wares produced in Korea [9]. This appears to occur at the Bronze age. Tempeh, miso and natto fermentations allow to improve soybeans taste and to increase the vitamins of B group [26]. This could compensate the losses of these water-soluble micronutrients during water treatments. Interestingly, Puri et al [27] mentioned that soybean sterilization was mandatory before inoculation of soy-mater and fermentation. They reported that boiling considerably reduced IFs in subsequent fermented soy-food. Sterilization via boiling was the process traditionally applied in ancient time. Water contact with seeds favored the leakage of glycosylated IFs into the cooking water. Nowadays, to save time and energy, sterilization processes are mainly performed by steaming under pressure using an autoclave [25,28]. However, it appears that steaming reduces the contact between soy-mater and water maintained larger amounts of IFs in final foodstuffs [29]. Consequently, the estrogenic IFs levels are higher in steamed food than in boiled food. And while this effect may be beneficial when IFs are required and useful, it constitutes a risk when they reach excessive levels.

4.3. Health Effects

4.3.1. Traditional Pharmacopeia

Soy polyphenol content has been studied thoroughly. So, soybean is known to contain many functional substances such as phenolic acids, flavonoids, IFs, stilbenes, saponins [26] or the peptide lunasin. According to Baraibar Norberg and Deutsh [7] soybean appeared first in the traditional Chinese pharmacopeia. The oldest treaties reporting soy medicinal properties still available is the *Shiliao bencao* (Compendium of Diet Therapy [Materia Dietetica]), which was written by Meng Shen in 670 CE. It can be read there that "*soybeans boiled into a liquid form, can eradicate poisons from the system and cure gastric fever, paralysis, pains, difficulty in passage of urine and other bladder troubles. It can also improve circulation of the blood, improper heart, liver, kidneys and stomach function and even remedy the chills*" [7,30]. It should be noted that none of these health effects rely on estrogenic properties. This observation suggests that IFs were not present in these preparations confirming that traditional processes tended to eliminate them. By comparison, kudzu roots which contains GEN, DAI and puerarin among other substances, were known to induce some estrogenic and antipyretic effects. The latter are now attributed to IFs [31] and suggest that the traditional preparation of kudzu preserved GEN and DAI levels [31]. These facts put together with data obtained on IFs content of soy-foods prepared following traditional recipes, strongly suggest that the exposure to IFs was low in pre-industrialized period. It most probably increased in Human populations with industrial processing.

4.3.2. Toxic Effects and Reference Doses

The reprotoxic effects of soy IFs and their methoxylated parents from clover were first documented in the 1940's when ewes were affected by the clover disease [32]. Within four years the

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herds' fertility was reduced from 80% to less than 5% and many farmers stopped their activities. Veterinaries then understood that IFs reduced the pituitary hormones FSH and LH which themselves were decreased due to an impairment of GnRH production at hypothalamic level [33]. Such mechanism is known to be used pharmacologically in contraceptive pills based on the synthetic estrogen: ethynyl-estradiol [34]. This impairment of pituitary hormones was then observed in premenopausal women who had 45 mg dietary IFs per day [35]. More recently it was shown that an exposure to 50 mg IFs per day significantly increases the risk of luteal deficiency [36] and infertility [37] in American women. An exposure of baby-girls to soy IFs via soy-based infant formulas was shown to increase the incidence of fibrosis, endometriosis, menstrual pains and bleedings [38–41]. These impairments were also observed in women with an over-consumption of soy-based products [42]. Moreover, a case of hypogonadism, gynecomastia and reduced libido was observed in a Japanese man over-consuming soy-milk for four years [43]. Finally, in five studies, soy IFs in biological fluids were associated to reduced quality and quantity of semen in men [44–48].

In addition, a controversy remains about the effects of IFs on breast cancers. An obvious discrepancy is observed between two data sources. On one hand, there are data obtained in vitro on recognized cell models [3,49], in animals implanted with these cancer cells [3,50], in healthy pre-menopausal women under IFs oral supplementation [3,51], and in women with breast cancer supplemented with soy and IFs [3,52]. On the other hand there are epidemiological studies [3,53] and review of randomized controlled trials (RCTs) [3,54]. The population studies tend to show a reduction of breast cancer risk in Asian women in an Asian environment (including green tea polyphenols intake) but this protection is not clearly observed in Western women in a Western environment [3,55]. Many biases including genetic, dietary and environmental parameters, make it difficult to reach a definitive conclusion on the effects of IFs on breast cancer incidence in Western menopausal women. The reviews including RCTs concluded that there was no increased risk of breast cancer in women taking soy or food supplements based on soy IFs [3,56]. However, the subjects recruited for these RCTs were selected on health criteria and did not represent the real population. Moreover, the RCTs were not designed to study the incidence of breast cancers and thus the conclusions of these analyses should be considered cautiously.

Still in humans, there are now enough evidences that soy-IFs can interact with thyroid function. On top of mechanistic arguments [3], clinical cases [57,58], observation studies [59,60] and intervention studies [61] confirm that soy IFs have slight anti-thyroid effects and can worsen the status of hypothyroid patients.

Beside these effects directly reported in human beings, the USA NTP showed that GEN was reprotoxic in sprag Dawley rats in a multi-generational study published in 2008 [4]. The study showed that some reproductive parameters including anogenital distance in pups and litter size were altered at a GEN dose of 35 mg/kg/day in males. According to the general toxicology rules, the NTP study can be used to define a Reference Dose in human, by applying safety factors to the level defined. Indeed, an amount of 35 $mg.kg^{-1}day^{-1}$ is a LOAEL (Lowest Observed Adverse Effect Level) and no effect were recorded on reproductive parameters at the dose of 7 $mg.kg^{-1}.day^{-1}$. When a LOAEL is available, the safety factors to apply should be 10 for interspecific differences between rat and human, 10 for intraspecific differences i.e. interindividual differences between human beings and 1.8 or 3 for conversion of LOAEL to NOAEL. If the last factor is fixed at 1.8, the Reference Dose for GEN appears to be about 20 $mg.day^{-1}$ for an adult of 60 kg. In addition, as GEN is usually present in food with DAI, both IFs should be considered. Unfortunately, there is neither LOAEL nor NOAEL for DAI in rats. Also, even if DAI is less estrogenic than GEN, it can be converted into Equol which may be more active than GEN on certain tissues [3]. Consequently, it would be relevant to consider the limit concentration of both GEN and DAI to be set at 20 $mg.day^{-1}$ for an adult of 60 kg. The present study show that the IFs levels reached in tofu, tempeh, miso and soy-juice are below this limit after water treatments meanwhile this is not the case in the corresponding industrial products. For textured proteins, the treatments allowed to decrease IFs concentrations from 35 to 22 mg/100g depending on protein sizes. This is higher than the potential reference dose. However, textured proteins are usually used with other ingredients and at a percentage ranging from 45 to 20%.

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Therefore, the final concentration in a 100g portion is lower than 20 mg. This dose is about half that which was shown to have a physiological effect in humans and seems to correspond to exposure levels in Asian populations following traditional soy-food preparation processes. Finally, these various doses discussed here correspond to median modern exposures in China and Japan [15,16]. To our knowledge, thy did not induce deleterious effects in the past.

To conclude this part, excess IFs should be avoided by populations which should not be exposed to estrogens. These are infants, children, premenopausal women, pregnant women, hypothyroid patients and men. Concerning women with breast cancer, it seems difficult to advice soy-consumption considering the estrogenic effects of IFs. The latter may prevent from breast cancer occurrence but their actions on an established breast cancer dependent on estrogens are still a subject of debate.

4.3.3. Beneficial Health Effects for a Restricted Population

If IFs should be carefully monitored it is because they seem to be active in human beings at dietary levels. Their beneficial estrogenic effects are restricted to certain categories of populations. Indeed, the most studied effect is the reduction of menopausal symptoms. The last meta-analysis which analyzed the effects of IFs on vasomotor symptoms and criteria showed only modest effects [62]. However other meta-analyses reported a decrease in hot-flushes occurrence [63]. In some studies, the effects appeared to be restricted to some natural compounds such as Equol [64] while in others, only some preparation-types were found to be active [65]. Nevertheless, while latest studies tend to show that vasomotor symptoms equally affect Asian and Western peri- and post-menopausal women [66] in accordance with an Asian exposure to IFs lower than 50 mg/day, IFs are the most popular substances used world-wide to reduce hot-flushes.

Besides menopausal symptoms, there are evidences that IFs doses >80 mg /day can prevent Bone Mineral Density (BMD) decrease in menopausal women [67]. Equol is also considered to have a specific beneficial effect on BMD during menopause when administered as supplement [68]. In the latter study, a RCT gathered 76 menopausal women between 50 and 55 years-old, the treatment lasted one year and the supplement contained 80 mg IFs aglycone, 10 mg of Equol and 25 mg Resveratrol as an antioxidant. In these specific conditions the BMD was preserved significantly in the treated group compared to the placebo group.

To conclude, there are enough data showing that IFs may be useful for menopausal women when there is no thyroid or estrogen-dependent diseases.

4.4. Consequences of This Finding on the Estimation of the Populations Exposure

This study has shown that simple water treatments like water rinsing, soaking or boiling can dramatically reduce IFs from soy-foods. It also gathered historical testimonies indicating that such water treatments were traditional to prepare soy-food in Asia as soybeans were increasingly eaten. The historical data shows that fermentation was developed quite early to improve soy digestibility, taste and preservation. Looking at actual recipes reported by Asian consumers, it appears that homemade soy is generally processed following family recipes and thus using water treatments. However, nowadays traditional boiling in water, tends to be replaced by steaming which reduces water-to-beans contact and maintain IFs in soy-foods.

Hence, from the most significant result of this work is the large differences observed between water treated products and those found on the market due to limited water treatments in industry to reduce energy and environmental costs. Thus, the importance to check the origin of soy-foods when estimating IFs intakes is highlighted. This is particularly true in Asia where a larger percentage of people may be prompt to prepare soy at home following family recipes. Table 2 below shows the discrepancies between IFs plasma levels as found in different studies where IFs intakes were estimated.

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Table 2. Review of isoflavones concentrations in human plasma in relation to estimated isoflavone intakes.

Location	Estimation method	Estimated IFs Intake (mg)	IFs Plasma (nM)	Time of plasma collection	Study
Los Angeles	Hawaii Food Composition Database based on commercial items	23.72	30.1	No specific instruction	[69]
China (Shanghai)	USDA-ISU database based on commercial items	23.5 58.15 84.5 284.0	106.3 119.9 146.3 188.3	No specific instruction	[70]
Japan	From [71] (commercial items)	46.4	419.2	Overnight fasting	[72]
Japan	From [71] (commercial items)	40.8	411.0	After fasting > 5h	[73]
England	USDA-ISU database based on commercial items	33.7	510	Not precise	[74]
Japan	From [71] (commercial items)	32.5 34.8	553* 605*	No specific instruction	[75]
The Netherland	Strictly controlled soy protein diet	48.5 100.1 104.2 93.9	2160 2530 2900 4800	Fasting samples after chronic intake	[76]
Hawaii	USDA-ISU database based on commercial items	96.0	5600	12h fasting and chronic intake	[77]

* Figures calculated from values experssed in $ng.mL^{-1}$ from [75].

As can be seen in Table 2, plasma IFs levels are much higher in Western people under an equivalent estimation of IFs exposure. These latter were generally assessed by assaying commercial soy-foods found on the corresponding local markets. Of course, at least part of the differences may come from analytical techniques and blood sampling time. However, in Asia, if IFs are ingested on a regular basis, like every day, a steady-state level may be expected. Therefore, whatever the time of sampling, blood levels should be fairly stable. On the opposite, in Western countries, where soy intake remains irregular, the time of sampling plays a major role on blood IFs concentrations and this may explain the low blood levels recorded in [74]. In addition, in [14] it was shown that there are no fundamental differences in IFs metabolism and pharmacokinetic between Asian and Western consumers. Thus, the intake estimation should be assessed to explain the discrepancies appearing in Table 2. While it is likely that in the West, soy-food come mainly from industrial sources as there is no tradition of soy-cooking, in Asia it is customary to prepare soy at home following family recipes. Therefore, the intake estimation may be over-evaluated in Asia if it is only based on industrial products as in Kimira *et al*. [71] or in Chan *et al.* [78] works. This comment should be considered while discussing about a Reference Dose for soy-IFs.

5. Conclusion

In population studies, the distinction between domestically and industrially processed soy-foods has not been made, potentially leading to an overestimation of overall exposure of soy-consumers to IFs. This is particularly true in Asian contexts where traditional soy-cooking practices prevail. A comprehensive review of traditional soy-processing methods reveals a consistent practice: soybeans were invariably boiled, either as a precursor to subsequent fermentation steps or to enhance their digestibility. This study shows that traditional and domestic processing methods significantly reduce the content of IFs in soy based-foods when compared to industrial methods. The application of two or three water treatments aids in achieving the potential “Reference Dose” for IFs, established according to the toxicological studies of the US National Toxicology Program. Furthermore, the results corroborate previous findings that water treatments can significantly decrease IFs in soy-foods.

Given the functional activities of IFs, their use should be judicious. One approach to achieve this goal is by controlling dietary exposure levels through the introduction of water treatments at both industrial and domestic soy-food preparation stages, thereby ensuring a safer democratization of their consumption. This simple rule could indeed result in a substantial shift in the global human exposure to estrogenic substances.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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References

1. Lee A, Bensaada S, Lamothe V, Lacoste M, Bennetau-Pelissero C. Endocrine disruptors on and in fruits and vegetables: Estimation of the potential exposure of the French population. *Food Chem.* **2022**, *373*, 131513–24.
2. Gier K, Preininger C, Sauer U. A Chip for Estrogen Receptor Action: Detection of Biomarkers Released by MCF-7 Cells through Estrogenic and Anti-Estrogenic Effects. *Sensors (Basel)*. **2017**, *17*, 1760.
3. Canivenc-Lavier MC, Bennetau-Pelissero C. Phytoestrogens and Health Effects. *Nutrients*. **2023**, *15*, 317.
4. NTP. Multigenerational reproductive study of genistein (Cas No. 446-72-0) in Sprague-Dawley rats (feed study). *Natl Toxicol Program Tech Rep Ser.* **2008**, *539*, 1–266.
5. NTP. Toxicology and carcinogenesis studies of genistein (Cas No. 446-72-0) in Sprague-Dawley rats (feed study). *Natl Toxicol Program Tech Rep Ser.* **2008**, *545*, 1–240.
6. Cao ZH, Green-Johnson JM, Buckley ND, Lin QY. Bioactivity of soy-based fermented foods: A review. *Biotechnol Adv*. **2019**, *37*, 223–238.
7. Baraibar Norberg M, Deutsh L. The first soybean cycle (domestication to 900 CE). In *The Soybean Through World History. Lessons for Sustainable Agrofood Systems* 1st ed Routledge studies in Food, Society and the Environment Taylor & Francis UK, 2023; pp: 22-56.
8. Zhao Z. New Archaeobotanic Data for the Study of the Origins of Agriculture in China. *Current Anthropology*. **2011**, *52*, 295–306.
9. Lee C-H, Kim ML. History of Fermented Foods in Northeast Asia. In *Ethnic Fermented Foods and Alcoholic Beverages of Asia* Tamang JP. Ed Spinger India 2016; pp: 1-16.
10. Lander B, DuBois TDA. History of Soy in China: From Weedy Bean to Global Commodity In Ethnic Fermented Foods and Alcoholic Beverages of Asia In The age of the soybean: An environmental history of soy During the great acceleration da Silva CM, de Majo C. Eds 2022: pp 29-47.
11. Traditional Tofu making experience in Debao Guangxi. Available online: https://twojadebowls.com/2013/08/16/traditional-tofu-making-experience-in-debao-guangxi/ (accessed on 27 February 2024)
12. Bensaada S, Chabrier F, Ginisty P, Ferrand C, Peruzzi G, et al. Improved Food-Processing Techniques to Reduce Isoflavones in Soy-Based Foodstuffs. *Foods.* **2023**, *12*, 1540–1557.
13. McClure T, Cocuron JC, Osmark V, McHale LK, Alonso AP. Impact of Environment on the Biomass Composition of Soybean (Glycine max) seeds. *J Agric Food Chem.* **2017**, *65*, 6753–6761.

14. Vergne S, Sauvant P, Lamothe V, Chantre P, Asselineau J, et al. Influence of ethnic origin (Asian v. Caucasian) and background diet on the bioavailability of dietary isoflavones.*Br J Nutr*. **2009**, *102*, 1642–1653.
15. Zhu J, Zhao Q, Qiu Y, Zhang Y, Cui S, et al. Soy Isoflavones Intake and Obesity in Chinese Adults: A Cross-Sectional Study in Shang-hai, China. *Nutrients.* **2021**, *13*, 2715. https://doi.org/10.3390/nu13082715.
16. Murai U, Sawada N, Charvat H, Inoue M, Yasuda N, et al. Soy product intake and risk of incident disabling dementia: the JPHC Disabling Dementia Study. *Eur J Nutr.* **2022**, *61*, 4045–4057.
17. Fernandez-Lopez A, Lamothe V, Delample M, Denayrolles M, Bennetau-Pelissero C. Removing isoflavones from modern soyfood: Why and how? *Food Chem.* **2016**, *210*, 286–294.
18. The Japanese Lab. How miso was made traditionally https://thejapanesefoodlab.com/traditional-japanese-miso#how / (accessed on 27 February 2024)
19. Vergne S, Titier K, Bernard V, Asselineau J, Durand M, et al. Bioavailability and Urinary Excretion of Isoflavones in Humans: Effects of Soy-Based Supplements Formulation and Equol Production. *J Pharm Biomed Anal.* **2007**, *43*, 1488–1494.
20. Shinkaruk S, Durand M, Lamothe V, Carpaye A, Martinet A, et al. Bioavailability of Glycitein Relatively to Other Soy Isoflavones in Healthy Young Caucasian Men. *Food Chem.* **2012**, *135*, 1104–1111.
21. Bennetau-Pelissero C, Le Houérou C, Lamothe V, Le Menn F, Babin P, et al. Synthesis of Haptens and Conjugates for ELISAs of Phytoestrogens. Development of the Immunological Tests. *J Agric Food Chem.* **2000**, *48*, 305–311.
22. Le Houerou C, Bennetau-Pelissero C, Lamothe V, Le Menn F, Babin P, et al. Syntheses of Novel Hapten-Protein Conjugates for Production of Highly Specific Antibodies to Formononetin, Daidzein and Genistein. *Tetrahedron.* **2000**, *56*, 295–301.
23. Bennetau-Pelissero, C. Plant Proteins from Legumes. In: Mérillon, JM., Ramawat, K. (eds) Bioactive Molecules in Food Vol 1. Reference Series in Phytochemistry. Springer, Cham. 2018 pp. 223–66.
24. Afssa, Afssaps. Sécurité et bénéfices des phyto-estrogènes apportés par l'alimentation – Recommandations Rapport d'expertise Mars 2005.
25. Kao TH, Lu YF, Hsieh HC, Chen BH. Stability of isoflavone glucosides during processing of soymilk and tofu. *Food Res Int*. **2004**, *37*, 891–900.
26. Chung IM, Seo SH, Ahn JK, Kim SH. Effect of processing, fermentation, and aging treatment to content and profile of phenolic compounds in soybean seed, soy curd and soy paste. *Food Chem*. **2011**, *127*, 960–967.
27. Puri A, Mir SR, Panda BP. Effect of sequential bio-processing conditions on the content and composition of vitamin K2 and isoflavones in fermented soy food. *J Food Sci Technol.* **2015**, *52*, 8228–8235.
28. Qiao Y, Zhang K, Zhang Z, Zhang C, Sun Y, et al. Fermented soybean foods: A review of their functional components, mechanism of action and factors influencing their health benefits. *Food Res Int* **2022**, *158*, 111575.
29. Kuligowski M, Sobkowiak D, Polanowska K, Jasinska-Kuligowska I. Effect of different processing methods on isoflavone content in soybeans and soy products *J Food Comp Anal* **2022**, *110*, 104535–41.
30. Shurtleff W, Aoyagi A. Early History of Soybeans and Soyfoods Worldwide (1024 BCE to 1899): Extensively Annotated Bibliography and Sourcebook. Lafayette, CA: *Soyinfo Center*. **2014**.
31. Wang S, Zhang S, Wang S, Gao P, Dai L. A comprehensive review on Pueraria: Insights on its chemistry and medicinal value. *Biomed Pharmacother.* **2020**, *131*, 110734–51.
32. Bennetts HW, Underwood EJ, Shier FL. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust Vet J.* **1946**, *23*, 2–13.
33. Findlay JK, Buckmaster JM, Chamley WA, Cumming IA, Hearnshaw H; et al. Release of luteinising hormone by œstradiol 17β and a gonadotrophin-releasing hormone in ewes affected with clover disease. *Neuroendocrinol*. **1973**, *11*, 57–66.
34. Cohen BL, Katz M. Further studies on pituitary and ovarian function in women receiving hormonal contraception. *Contraception*. **1981**, *24*, 159–172.
35. Cassidy A, Bingham S, Setchell KD. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr*. **1994**, *60*, 333–340.
36. Andrews MA, Schliep KC, Wactawski-Wende J, Stanford JB, Zarek SM, et al. Dietary factors and luteal phase deficiency in healthy eumenorrheic women. *Hum Reprod*. **2015**, *30*, 1942–1951.
37. Jacobsen BK, Jaceldo-Siegl K, Knutsen SF, Fan J, Oda K, et al. Soy isoflavone intake and the likelihood of ever becoming a mother: the Adventist Health Study-2 *Int J Womens Health*. **2014**, *6*, 377–384.
38. Strom BL, Schinnar R, Ziegler EE, Barnhart KT, Sammel MD, et al. Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood *JAMA*. **2001**, *286*, 807–814.
39. Upson K, Harmon QE, Laughlin-Tommaso SK, Umbach DM, Baird DD. Soy-based Infant Formula Feeding and Heavy Menstrual Bleeding Among Young African American Women. *Epidemiology*. **2016**, *27*, 716–725.
40. Upson K, Adgent MA, Wegienka G, Baird DD. Soy-based infant formula feeding and menstrual pain in a cohort of women aged 23-35 years. *Hum Reprod*. **2019**, *34*, 148–154.

41. Qin H, Lin Z, Vásquez E, Luan X, Guo F, Xu L. High soy isoflavone or soy-based food intake during infancy and in adulthood is associated with an increased risk of uterine fibroids in premenopausal women: a meta-analysis. *Nutr Res*. **2019**, *71*, 30–42.
42. Chandrareddy A, Muneyyirci-Delale O, McFarlane SI, Murad OM. Adverse effects of phytoestrogens on reproductive health: a report of three cases. *Complement Ther Clin Pract*. **2008**, *14*, 132–135.
43. Imai H, Nishikawa H, Suzuki A, Kodama E, Iida T, et al. Secondary Hypogonadism due to Excessive Ingestion of Isoflavone in a Man. *Intern Med*. **2022**, *26*.
44. Chavarro JE, Toth TL, Sadio SM, Hauser R. Soy food and isoflavone intake in relation to semen quality parameters among men from an infertility clinic. *Hum Reprod*. **2008**, *23*, 2584–2590.
45. Toshima H, Suzuki Y, Imai K, Yoshinaga J, Shiraishi H, et al. Endocrine disrupting chemicals in urine of Japanese male partners of subfertile couples: a pilot study on exposure and semen quality. *Int J Hyg Environ Health*. **2012**, *215*, 502–506.
46. Xia Y, Chen M, Zhu P, Lu C, Fu G, et al. Urinary phytoestrogen levels related to idiopathic male infertility in Chinese men. *Environ Int*. **2013**, *59*, 161–167.
47. Mumford SL, Kim S, Chen Z, Boyd Barr D, Buck Louis GM. Urinary Phytoestrogens Are Associated with Subtle Indicators of Semen Quality among Male Partners of Couples Desiring Pregnancy. *J Nutr*. **2015**, *145*, 2535–2541.
48. Yuan G, Liu Y, Liu G, Wei L, Wen Y, et al. Associations between semen phytoestrogens concentrations and semen quality in Chinese men. *Environ Int*. **2019**, *129*, 136–144.
49. Schmitt E, Dekant W, Stopper H. Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. *Toxicol In Vitro*. **2001**, *15*, 433–439.
50. Wu Q, Yang Y, Yu J, Jin N. Soy isoflavone extracts stimulate the growth of nude mouse xenografts bearing estrogen-dependent human breast cancer cells (MCF-7). *J Biomed Res*. **2012**, *26*, 44–52.
51. McMichael-Phillips DF, Harding C, Morton M, Roberts SA, Howell A, et al. Effects of soy-protein supplementation on epithelial proliferation in the histologically normal human breast. *Am J Clin Nutr*. **1998**, *68*, 1431S-5S.
52. Shike M, Doane AS, Russo L, Cabal R, Reis-Filho JS, et al. The effects of soy supplementation on gene expression in breast cancer: a randomized placebo-controlled study. *J Natl Cancer Inst*. **2014**, *106*, dju 189.
53. van Die MD, Bone KM, Visvanathan K, Kyrø C, Aune D, et al. Phytonutrients and outcomes following breast cancer: a systematic review and meta-analysis of observational studies. *JNCI Cancer Spectr*. **2024**, *8*, pkad104.
54. Messina M. Impact of Soy Foods on the Development of Breast Cancer and the Prognosis of Breast Cancer Patients. *Forsch Komplementmed*. **2016**, *23*, 75–80.
55. Chen M, Rao Y, Zheng Y, Wei S, Li Y, et al. Association between soy isoflavone intake and breast cancer risk for pre- and post-menopausal women: a meta-analysis of epidemiological studies. *PLoS One*. **2014**, *9*, e89288.
56. Finkeldey L, Schmitz E, Ellinger S. Effect of the Intake of Isoflavones on Risk Factors of Breast Cancer-A Systematic Review of Randomized Controlled Intervention Studies. *Nutrients*. **2021**, *13*, 2309.
57. Caprio AM, Umano GR, Luongo C, Aiello F, Dello Iacono I, et al. Case report: Goiter and overt hypothyroidism in an iodine-deficient toddler on soy milk and hypoallergenic diet. *Front Endocrinol* (Lausanne). **2022**, *13*, 927726.
58. Fruzza AG, Demeterco-Berggren C, Jones KL. Unawareness of the effects of soy intake on the management of congenital hypothyroidism. *Pediatrics*. **2012**, *130*, e699-702.
59. Conrad SC, Chiu H, Silverman BL. Soy formula complicates management of congenital hypothyroidism. *Arch Dis Child*. **2004**, *89*, 37–40.
60. Fan Y, Qian H, Wu Z, Li Z, Li X, et al. Exploratory analysis of the associations between urinary phytoestrogens and thyroid hormones among adolescents and adults in the United States: National Health and Nutrition Examination Survey 2007-2010. *Environ Sci Pollut Res Int*. **2022**, *29*, 2974–2984.
61. Sathyapalan T, Manuchehri AM, Thatcher NJ, Rigby AS, Chapman T, et al. The effect of soy phytoestrogen supplementation on thyroid status and cardiovascular risk markers in patients with subclinical hypothyroidism: a randomized, double-blind, crossover study. *J Clin Endocrinol Metab*. **2011**, *96*, 1442–1449.
62. Guo PP, Li P, Zhang XH, Liu N, Wang J, et al. Complementary and alternative medicine for natural and treatment-induced vasomotor symptoms: An overview of systematic reviews and meta-analyses. *Complement Ther Clin Pract*. **2019**, *36*, 181–194.
63. Chen MN, Lin CC, Liu CF. Efficacy of phytoestrogens for menopausal symptoms: a meta-analysis and systematic review. *Climacteric*. **2015**, *18*, 260–269.
64. Daily JW, Ko BS, Ryuk J, Liu M, Zhang W, et al. Equol Decreases Hot Flashes in Postmenopausal Women: A Systematic Review and Meta-Analysis of Randomized Clinical Trials. *J Med Food*. **2019**, *22*, 127–139.
65. Taku K, Melby MK, Kronenberg F, Kurzer MS, Messina M. Extracted or synthesized soybean isoflavones reduce menopausal hot flash frequency and severity: systematic review and meta-analysis of randomized controlled trials. *Menopause*. **2012**, *19*, 776–790.

 doi:10.20944/preprints202403.0708.v1

66. Islam RM, Bell RJ, Rizvi F, Davis SR. Vasomotor symptoms in women in Asia appear comparable with women in Western countries: a systematic review. *Menopause.* **2017**, *24*, 1313–1322.
67. Taku K, Melby MK, Takebayashi J, Mizuno S, Ishimi Y, et al. Effect of soy isoflavone extract supplements on bone mineral density in menopausal women: meta-analysis of randomized controlled trials *Asia Pac J Clin Nutr*. **2010**, *19*, 33–42.
68. Corbi G, Nobile V, Conti V, Cannavo A, Sorrenti V, et al. Equol and Resveratrol Improve Bone Turnover Biomarkers in Postmenopausal Women: A Clinical Trial. *Int J Mol Sci*. **2023**, *24*, 12063.
69. Wu AH, Yu MC, Tseng CC, Twaddle NC, Doerge DR. Plasma isoflavone levels versus self-reported soy isoflavone levels in Asian-American women in Los Angeles County. *Carcinogenesis*. **2004**, *25*, 77–81.
70. Frankenfeld CL, Lampe JW, Shannon J, Gao DL, Ray RM, et al. Frequency of soy food consumption and serum isoflavone concentrations among Chinese women in Shanghai. *Public Health Nutr.* **2004**, *7*, 765–772.
71. Kimira M, Arai Y, Shimoi K, Watanabe S. Japanese intake of flavonoids and isoflavonoids from foods. *J Epidemiol*. **1998**, *8*, 168–175.
72. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, et al. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *J Nutr.* **2000**, *130*, 2243–2250.
73. Yamamoto S, Sobue T, Sasaki S, Kobayashi M, Arai Y, et al. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a japanese population in comparison with dietary records and blood and urine isoflavones. *J Nutr.* **2001**, *131*, 2741–2747.
74. Verkasalo PK, Appleby PN, Allen NE, Davey G, Adlercreutz H, et al. Soya intake and plasma concentrations of daidzein and genistein: validity of dietary assessment among eighty British women (Oxford arm of the European Prospective Investigation into Cancer and Nutrition). *Br J Nutr*. **2001**, *86*, 415–421.
75. Iwasaki M, Inoue M, Otani T, Sasazuki S, Kurahashi N, et al. Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case-control study from the Japan Public Health Center-based prospective study group. *J Clin Oncol*. **2008**, *26*, 1677–1683.
76. van der Velpen V, Hollman PC, van Nielen M, Schouten EG, Mensink M, et al. Large inter-individual variation in isoflavone plasma concentration limits use of isoflavone intake data for risk assessment. *Eur J Clin Nutr*. **2014**, *68*, 1141–1147.
77. Gardner CD, Chatterjee LM, Franke AA. Effects of isoflavone supplements vs. soy foods on blood concentrations of genistein and daidzein in adults. *J Nutr Biochem*. **2009**, *20*, 227–234.
78. Chan SG, Murphy PA, Ho SC, Kreiger N, Darlington G, et al. Isoflavonoid content of Hong Kong soy foods. *J Agric Food Chem*. **2009**, *57*, 5386–5390.