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Removing isoflavones from modern soyfood: Why and how?

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ABSTRACT

Estrogenic isoflavones were found, in the 1940s, to disrupt ewe reproduction and were identified in soy-consumers' urine in 1982. This led to controversy about their safety, often supported by current Asian diet measurements, but not by historical data. Traditional Asian recipes of soy were tested while assaying soy glycosilated isoflavones. As these compounds are water-soluble, their concentration is reduced by soaking. Pre-cooking or simmering time-dependently reduces the isoflavone:protein ratio in Tofu. Cooking soy-juice for 15 or 60 min decreases the isoflavone:protein ratios in Tofu from 6.90 to 3.57 and 1.80, respectively ($p < 0.001$). Traditional Tempeh contains only 18.07% of the original soybean isoflavones ($p < 0.001$). Soy-juice isoflavones were reduced by ultra-filtration (6.54 *vs* 1.24 isoflavone:protein; $p < 0.001$). Soy-protein and isoflavones are dissociated by water rinsing and prolonged cooking, but these have no equivalent in modern processes. As regards human health, a precise definition of the safety level of isoflavone intake requires additional studies.

1. Introduction

Soy has constituted a significant part of the Western human and animal diet since its industrialised processing started in the 1940s (Barnes, 2010, with references). Nowadays, soy isoflavones have undoubtedly become the most prevalent and potent xenoestrogens in human food (Omoruyi, Kabiersch, & Pohjanvirta, 2013). Xenoestrogens are known to impair reproduction efficiency. It has been argued since the late 1950s, that reproductive endocrine disruption could reduce human fertility, resulting in fewer births in industrialised countries (Lang & Nuevo-Chiquero, 2012). There are certainly many factors, ranging from environmental to socio-economic, involved in the lessened desire to have children, inducing subsequent demographic decline. However, several scientists are now hypothesising an adverse effect of environmental endocrine disruption that triggers reduced sperm count (Splingart et al., 2012), or increased incidence of spontaneous early miscarriages (Lang & Nuevo-Chiquero, 2012). If endocrine disruptors are, at least partly, responsible for this situation, then isoflavones, as the most prevalent xenoestrogens since the late 1950s in the human diet, should be considered as additional potential endocrine disruptors.

Genistein (**Gen**) and daidzein (**Daid**), the main estrogenic soy isoflavones, have been extensively studied since the 1960s, both for their beneficial and adverse effects (Barrett, 1996; Bennetau-Pelissero, 2013; Bondesson & Gustafsson, 2010). Both isoflavones are found in soy at several mg $100\,g^{-1}$ (Bennetau-Pelissero, 2013). The deleterious effects of these compounds, as metabolites of clover isoflavones, were first documented in 1946 by Bennetts and co-workers studying New Zealand ewes expressing clover disease, an infertility syndrome (Bennetts, Underwood, & Shier, 1946). When equol, a metabolite of **Daid**, was found in human urine in 1982 (Axelson et al., 1982), health concerns became focused on humans.

As early as 1984, Adlercreutz (Adlercreutz, 1984) suggested that soy isoflavones had beneficial effects, founding his argument on contemporaneous observations of Asian populations, proceeding on the assumption that isoflavones had always been part and parcel of the Asian diet. He hypothesised that isoflavones could act as anti-estrogens, thereby protecting Asian populations from estrogen-dependent diseases such as breast, prostate and colon cancer (Adlercreutz, 1984). However, it is common knowledge that

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the characteristics of an Asian diet differ from a Western one in numerous ways (Morinaka et al., 2013), with soy intake only representing a small proportion of this difference (Morinaka et al., 2013).

Since then, the scientific community has mostly tended to argue in favour of either the beneficial or deleterious effects of soy isoflavones (Barrett, 1996; Bondesson & Gustafsson, 2010). Estrogenic effects are now seen as being beneficial for men and women with steroid deficiencies, *i.e.* persons over 50. However, some deleterious effects could also be expected in certain types of cancer or early exposure.

Bearing this in mind, the role soy isoflavones play in current human health issues, possibly linked to endocrine-disruptive chemicals, warrants more detailed studies. Accordingly, it is crucial to consider isoflavone consumption in human populations, including the isoflavones contained in the soy added to Western food for nutritional, economic or technological reasons. The interpretation of present-day data would be impacted if it were admitted that soy isoflavones were only infrequently associated with older-style soyfood. In other words, their estrogenic effects, either beneficial or adverse depending on the physiological context, should be reaffirmed. It is well known that: 1) soy cannot be significantly ingested without undergoing a cooking process; 2) cooking processes have been progressively elaborated over the centuries in Asia (Barnes, 2010); 3) there are key differences between traditional and modern soyfood processing techniques (Barnes, 2010). The present study, therefore, examined the impact of several soy cooking stages on estrogenic isoflavone content respecting traditional recipes. A modern process, ultra-filtration, was tested on soy-juice, in order to determine its impact on isoflavone content.

2. Material and methods

2.1. Soy products

The soy products tested for traditional recipes came from local food suppliers. Their isoflavone content was assayed according to the technique described below. Soy-juice (18.82 ± 1.29 mg 100 mL^{-1} **Gen** and 12.17 ± 1.58 mg 100 mL^{-1} **Daid**) used in the ultra-filtration process came from *Nutrition & Nature*, which belongs to the *Nutrition & Santé* group (Revel, France – https://www.nutritionetsante.com/fr). The soy products were dehulled soybean seeds sold in bulk (61.2 ± 3.03 mg 100 g^{-1} **Gen** and 35.2 ± 19.08 mg 100 g^{-1} **Daid**), soy flakes sold in bulk (55.41 ± 3.89 mg 100 g^{-1} **Gen** and 25.73 ± 1.46 mg 100 g^{-1} **Daid**), *Celnat* soy flour (*Celnat*, Saint-Germain-Laprade, France, batch RF: 32 413 – www.celnat.fr) (55.3 ± 3.56 mg 100 g^{-1} **Gen** and 25.73 ± 1.46 mg 100 g^{-1} **Daid**), UHT soy-juice (20.45 ± 0.65 mg 100 mL^{-1} **Gen** and 10.98 ± 0.27 mg 100 mL^{-1} **Daid**) (*Biosoy*, Soy, Nutrition & Nature, Revel, France, Batch: T006 T 346 7 13:51 012 – https://www.soy.fr/boissons-vegetales/biosoy-nature) and Tempeh (29.23 ± 1.21 mg 100 g^{-1} **Gen** and 16.08 ± 0.29 mg 100 g^{-1} **Daid**) (*Lima*, Batch 50.85.56 Aalter, Belgium – www.limafood.com). The home-made Tempeh was prepared by Nurlaili Robert (http://biosegar.com/biosegar.htm). In this document, isoflavone quantities are expressed in aglycone equivalent because of the enzymatic cleavage step used prior to the assay. Because de-conjugation is known to occur at gut level, expressing isoflavones as aglycones makes sense when either human or animal exposure is considered. All products, including Nigari ($MgCl_2$ – *Celnat*, Saint-Germain-Laprade, France, batch 14114, – www.celnat.com), were dietary grade foodstuff and were purchased from an organic market (*Biocoop* Pessac, Gironde, France – http://www.biocoop.fr/magasins-bio/Trouver-mon-magasin-Biocoop/Aquitaine/Gironde/SAVEURS-ET-NATURE-2).

2.2. Chemicals

The sodium bicarbonate was purchased as food grade salt from a local supermarket (*Casino*, Talence, France – http://www.supercasino.fr/-bordeaux-talence-.html). All other compounds were from *Sigma-Aldrich* (L'Isle d'Abeau Chesne, France), unless otherwise stated. β-glucuronidase-aryl sulfatase was from *Roche* (Basel, Switzerland). The secondary antibody came from *Dako* (Les Ulis, France) (ref. P0217). ELISA antibodies were obtained in our laboratory, and were used as described below.

2.3. Ultra-filtration procedure

Ultra-filtration steps were performed at the AGIR Food Industry Platform (Pessac, France) on a TIA pilot apparatus with a loading capacity of 50 L, and working with ceramic filtrating membranes 23–25 mm in diameter and 850 mm long. Filtration parameters were adjusted to the best isoflavone removal efficiency based on isoflavone ELISA measurements.

2.4. Cooking tests on soy-juice

The experimental procedures followed in this study for soy-juice and for Tofu are summarized in Supplementary Figs. S1A and S1B. The hypothesis was that prolonged cooking and extensive contact surface between soy protein and water would result in the increased leaking of isoflavones into the cooking water. Therefore, different cooking times were tested on different soybean foodstuff. All tests were made at least in triplicate.

2.4.1. Soy-juice preparation from dehulled soybean seed, whether pounded or unpounded

Two grams of dehulled soybean seeds (either pounded or unpounded) were put in 20 mL of distilled water and soaked for 20 h at room temperature. The soaked grains were either left uncooked or were pre-cooked at 90 °C for 5 min, 15 min, 30 min or 60 min. The water was removed and kept for isoflavone assay. Soaked beans, pre-cooked or not, were ground and re-suspended by stirring for 5 min in 14 mL of distilled water. The mixture was additionally cooked to reach a total cooking time of 60 min. It was filtered through a metal grid coated with a fabric which collected Okara. The filtered soy-juice was collected and a 1 mL sample was kept for isoflavone assay (see Fig. S1A for details).

2.4.2. Soy-juice preparation from soy-flakes, whether pounded or unpounded

Two grams of soybean flakes, either pounded or unpounded, were put in 20 mL of distilled water and soaked for 20 h at room temperature. The soaked flakes were either left uncooked or were pre-cooked at 90 °C for 5 min, 15 min, 30 min or 60 min. The water was removed and kept for isoflavone assay. Soaked flakes (pounded or unpounded), pre-cooked or not, were ground and re-suspended by stirring for 5 min in 14 mL of distilled water. The mixture was additionally cooked to reach a total cooking time of 60 min. It was filtered through a metal grid coated with a specifically designed fabric. The filtered soy-juice was collected and a 1 mL sample was kept for isoflavone assay (see Fig. S1A for details).

2.4.3. Soy-juice ultra-filtration

Fifteen litres of soy-juice prepared by the supplier *Nutrition & Santé* were mixed with 30 L of pre-filtered tap water, and heated to 50 °C. Twenty grams of $NaHCO_3$ per litre were then added. The heated diluted soy-juice was poured into an experimental ultra-filtration apparatus (TIA) and filtered at 400 kPa through a 5 KDa-pore-size membrane at a temperature of 55 °C.

Ultra-filtration parameters were adjusted by progressive changes driven by the isoflavone measurements in the resulting soy-juice.

2.5. Tofu preparation

2.5.1. Experimental Tofu preparation

In this case, Tofu was not obtained by pressing in a Tofu maker, but by centrifugation (Fig. S1B). The hypothesis was that the longer the cooking of the initial soy-juice, the higher the isoflavone transfer rate into the water fraction (Fig. S1B). Therefore, the duration of the soy-juice cooking step was tested for 4 cooking durations: 3 min, 15 min, 30 min, 60 min. Five mL of soy-juice was heated in a screw-capped 10 mL Pyrex glass-tube, using a heating bath set at 90 °C. After the different cooking durations, coagulation was obtained using 0.125 mL of Nigari solution (210 g L^{-1}). The final Nigari concentration was then 5.25 g L^{-1}. The mixture was swirled three times to mix the salt and juice, and put directly onto ice until centrifugation. The curd obtained by adding Nigari was spun for 10 min, at 4 °C, 3000*g*. The supernatant and curd were collected for isoflavone assay (see Fig. S1B for details).

To evaluate the impact of experimental rinsing on Tofu isoflavone concentrations, 5 mL of soy-juice was placed in a Pyrex glass tube and heated for 1 min to boiling point in a heating bath. One hundred and twenty-five µL of Nigari solution (final concentration 5.25 g L^{-1}) was added, and the resulting mixture was shaken very gently. Samples were cooled on ice. Tubes were centrifuged for 10 min, 4 °C and 3000*g*. The whey was collected and its volume measured. An original set of three tubes was kept for measurements and the procedure was then continued on 3 additional tubes. A volume of water equivalent to that of the previously collected whey was added, and the curd was re-suspended gently before a second centrifugation. This procedure was repeated in order to obtain an experimental Tofu curd that was rinsed 3 times. Tofu samples were collected for isoflavone analysis. The samples rinsed 3 times were compared to those obtained after simple curd formation and to the initial soy-juice.

2.5.2. Traditional Tofu preparation

Tofu was prepared from different soy-matter in accordance with traditional recipes and using a traditional Tofu maker (Midzu Kit fabrication-tofu). In the first preparation, Tofu was prepared from Biosoy UHT soy-juice, and curd-rinsing steps were included. Two rinsing procedures were tested: the first included only one rinsing-step, the other included 7 rinsing-steps, as reported in Barrett's review (Barrett, 1996). In a second recipe, Tofu was no longer prepared from soy-juice but from soy flour. This recipe is described by Wentland (2013), and is still used in the region of Debao-Guangxi in China. For the second recipe, the duration of the soy-meal cooking-step was modulated to determine to what extent the isoflavone removal had taken place.

To evaluate the impact of rinsing on Tofu isoflavone concentrations, 200 mL of soy-juice was heated to boiling point on a hot plate for 15 min. Ten mL of Nigari solution (final concentration 5.25 g L^{-1}) was added, and the resulting mixture was gently shaken. The curd was put directly on ice and kept on it until ready for the next step. The curd was poured onto a metal grid coated with specialist fabric, and the whey collected and its volume measured. The curd was put in a glass vial and a volume of distilled water, equivalent to the previously collected whey, was added. The curd was gently shaken for 15 s and then poured onto the metal grid coated with fabric, and the residual water was collected. The curd was then placed in a Tofu maker coated with the fabric and extra water was pressed out for 30 min. A Tofu sample was collected for isoflavone analysis. The same protocol was applied for a septuple rinsing procedure.

To evaluate the impact of cooking duration on the isoflavone content of Tofu made from soybean-meal in accordance with the Debao-Guangxi recipe, 285 mL of water was heated at 85 °C in a heating bath. One hundred grams of soybean meal was then gently shaken into the mixture. This was cooked for 5 min, 15 min, 30 min or 60 min at 85 °C in a heating bath. Fifteen mL of Nigari solution (final concentration 5.25 g L^{-1}) was added, and the mixture was shaken gently. The curd was put on ice until further processing, to ensure procedural consistency. It was then filtered through a metal grid covered by fabric. A sample of whey was kept for isoflavone measurements. The curd was put in a Tofu maker and pressed until there was no juice left. It was then kept pressed for an additional 30 min. The whey was collected and its volume measured. A sample of Tofu was collected for isoflavone measurement. The recipe was conducted in triplicate for each cooking time.

2.6. Tempeh preparation

Tempeh was prepared by an Indonesian migrant in accordance with a traditional recipe transmitted over several generations within her Indonesian family. Traditional Tempeh is obtained under tropical climate conditions using a specific ferment, *Rhizopus oligosporus*. This fungus was initially present on the hibiscus leaves used to wrap Tempeh for the fermentation step and then wrapped into banana leaves. In order to prevent the development of other fungi or bacteria during the fermentation step, the soybean seeds were traditionally rinsed and cooked several times in boiling water (natural sterilization and microbial reduction). Briefly, 200 g of dehulled soybean seeds was first rinsed 3 times in tap water. A first cooking step was performed in water until boiling point, with regular skimming for 20 min. The soybean seeds were then soaked for an additional 20 min in the heated cooking water. The soybean seeds were rinsed 3 times using tap water, and subsequently cooked for 20 min in boiling water. These were then left to soak in the heated water for an additional 20 min until made tender, as assessed by a finger crushing test. Once the beans had cooled, the water was removed and the beans dried in an oven at 80 °C. The resulting preparation was then cooled to room temperature, and the *Rhizopus oligosporus* (400 mg) ferment was added. The mixture was then placed into a 200 g sealed plastic storage bag, with aeration holes made using a fork. The incubation was carried out at 28 °C for 24 h before the Tempeh was wrapped in an additional plastic bag and vacuum-packed for conservation at 4 °C until assay.

2.7. Isoflavone assays

Gen and **Daid**, the two main isoflavones from soy, were assayed using two ELISA tools developed by our laboratory, and validated by an international ring test confronted with physico-chemical methods (Bennetau-Pelissero et al., 2003). All data are given in equivalent aglycone.

Briefly, the extraction was performed on samples diluted 1/50 in distilled water. For liquid matrices, 1 mL was diluted in 49 mL of water. For solid samples, 1 g was dispersed either ground or not in 49 mL of water. The resulting mixture was swirled for 15 min and 500 µL of the dilution was placed in a 10 mL Pyrex glass tube. For the digestion step, 1.5 mL of acetate buffer (0.5 M, pH 5, EDTA 40 mg L^{-1}, Penicillin G 45 mg L^{-1}, Streptomycin 25 mg L^{-1}) was added, followed by 10 µL of digestion enzyme (β-glucuronidase-arylsulfatase from *Helix pomatia*). Caps were then screwed onto the Pyrex tubes. The solution was incubated overnight at 37 °C with gentle swirling. A digestion control was run in parallel, using a solution of genistin (0.1 mg mL^{-1}). Four mL of ethyl acetate was added to the digestion solution. After a 15-s vortex step, the emulsion was separated by a 2 min spin at

4 °C, 3000*g*. The tubes were frozen for 60 min at −20 °C, and the organic phase was collected in a glass tube and then evaporated at room temperature until dry, using a speed-vac apparatus. The extraction was renewed twice on the same aqueous phase, with the three organic phases being pooled in the same collecting tube and then dried. The empty tubes then received 500 μL of assay buffer and the extract was re-suspended in the assay buffer by sonication. The samples were kept at −20 °C until assay. An extraction control prepared from a **Gen** solution (0.1 mg mL^{-1}) was run in parallel to check for extraction recovery. In all cases, the extraction rate was found to be between 96 and 102%.

The ELISAs respect competitive procedures with a fixed antigen coated in the microwells of the microtitration plates. The coated antigens were **Gen** and **Daid** haptens, coupled to swine thyroglobulin. These compounds were synthesized in our laboratory (Bennetau-Pelissero et al., 2003). The antibodies were specific to each isoflavone. They were raised in rabbits by our team to recognise haptens coupled to bovine serum albumin and their characteristics are indicated in a previous study (Bennetau-Pelissero et al., 2003). The sensitivity of the assays given as the mid-point of the standard curve was 8 ng $well^{-1}$ for **Daid** and 3.12 ng $well^{-1}$ for **Gen**. The detection limit was therefore 2 ng $well^{-1}$ for **Daid**, and 1.9 ng $well^{-1}$ for **Gen**. All samples had to be diluted to at least 1:500 to allow accurate determination compared to the standard curve. The inter-assay variations were 13.1% and 12.8% for **Gen** and **Daid**, respectively.

2.8. Statistical analysis

The data obtained were assumed to follow a normal distribution since no bias of any kind could be identified that would lead to another distribution. All experiments were analysed via an ANOVA test using the Statview software. ANOVA analyses were carried out on the data from each test within the same experiment, and for each of the different experiments. When a difference was recorded, the post hoc analysis was performed using non-parametric tests specially designed for small samples and following the Mann-Whitney procedure.

3. Results

3.1. Soy-juice preparation

3.1.1. Soy-juice from dehulled soybean seeds

The influence of pre-cooking duration (see Fig. S1A for details of the protocol) on dehulled soybean seeds is given in Fig. S2. This figure shows the impact of pre-cooking before bean crushing on isoflavone content in soy-juice. At this stage, the pre-cooking water was eliminated, along with any isoflavones it might contain. The subsequent cooking was designed to allow equal thermal treatment of the food matrix. If isoflavones passed into the water at the second cooking stage, they remained in the soy-juice and Okara. The juice prepared from the cooking mixture was separated from the Okara by simple filtration. The difference was highly significant ($p < 0.005$) in juice from soybean pre-cooked for 60 min when compared to juice from un-pre-cooked beans. Sixty minutes pre-cooking removed 54.32% of the initial isoflavones. With pre-cooking, the quantity of isoflavones increased in the soaking water, and in the Okara. It was significant in Okara ($p < 0.01$) when compared to the non-precooked and the 60 min pre-cooked samples. The results indicated, therefore, that the longer the pre-cooking step, the lower the isoflavone content in the resulting soy-juice. They also showed that when the cooking step is omitted just before filtration, the isoflavones remain in the Okara.

The influence of pre-cooking duration on the isoflavone content of soy-juice made from pounded dehulled beans is presented in Fig. 1. This figure shows the impact of a pre-cooking-step, included before pounded-soybean crushing, on isoflavone content in soy-juice. The pounded beans were expected to lose their isoflavones more easily because of a greater contact between seed and water. The graph shows that the greatest efficiency in isoflavone removal was obtained during the first 5 min. However, isoflavones were constantly being removed thereafter, although less efficiently, and the removal was related to the cooking duration. At the end of the 60 min cooking period, the removal percentages were 57.43% for Gen, 55.00% for Daid, and 56.68% for Gen + Daid.

3.1.2. Soy-juice from soy-bean flakes

The influence of pre-cooking duration on the isoflavone content of soy-juice made from flakes, pounded or un-pounded, is presented in Fig. 2. Pounding was expected to increase contact between the soy matrix and water and to increase isoflavone elimination in pre-cooking water. Fig. 2 shows that the isoflavone levels in juice obtained from flakes ground and pre-cooked for different durations are always lower than those from the corresponding juice made using entire flakes. However, the differences between pounded and un-pounded flakes became significant only after 60 min of cooking. At that time, the values were 13.87 ± 0.15 mg 100 g^{-1} *vs* 10.72 ± 0.32 mg 100 g^{-1} for un-pounded and pounded flakes, respectively. In both cases, however, the isoflavone content after 60 min cooking was significantly lower than in the initial soybean matrix (72.09% ± 0.78 *vs* 62.65% ± 1.89 in un-pounded and pounded flakes, respectively).

3.2. Soy juice ultra-filtration

The goal of the ultra-filtration procedure was to extract 80% of isoflavones from the initial soy-juice. Several attempts were performed to find an efficient protocol. The final combination, validated on 6 different tests, was as follows: temperature 55 °C, filtration cut-off 5 KDa, pressure 400 kPa. First, the diluted soy-juice (1 in 2 volumes of tap water) was filtered until the extra water was removed. Then, 10 L of pre-filtered extra tap water was added to the soy-juice and, once the water was eliminated, this procedure was repeated twice more. Samples were collected after the first step, and extra samples were collected at each water addition until the third and final rinsing step. In these conditions, the final isoflavone content was 18.79% ± 1.99% of the initial quantity (see Table 1 for more details). Fig. 3 gives the elimination kinetic

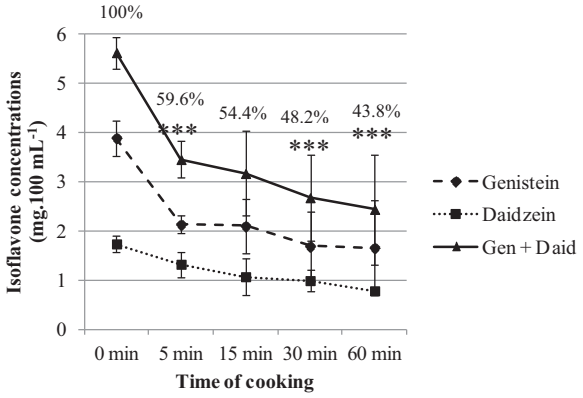

Fig. 1. Evolution of isoflavone concentrations of soy-juice based on pounded dehulled soybean seeds with time of pre-cooking. Percentages indicate the remaining isoflavones at each step. Error bars are SEM. *** indicate a significant difference with $p<0.005$ compared to initial isoflavone concentration.

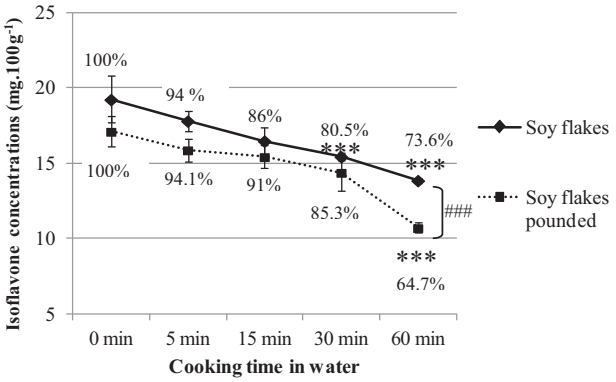

Fig. 2. Evolution of isoflavone concentrations in soy-juice made from soy-flakes pounded or not and pre-cooked in water for increasing durations. Percentages indicate the remaining isoflavones at each step. Error bars are SEM. *** indicate a significant difference with p<0.005 compared to initial isoflavone concentration. # indicates a significant difference between pounded and un-pounded flakes P<0.01.

of isoflavone with the initial content, the content at mid-process after the first elimination of added tap-water, and the final content after the 3 additional rinsing procedures. Table 1 gives isoflavone content from Tofu made from ultra-filtrated juice, showing that, when compared to the initial juice, the percentage of remaining isoflavone after Tofu processing was only 15.72%.

3.3. Tofu preparation

Tofu preparation was tested on experimental Tofu obtained in glass tubes by centrifugation or using a traditional process. This was done to evaluate the impact of curd rinsing on Tofu isoflavone content. According to other authors (Prabhakaran, Perera, & Valiyaveettil, 2006), Nigari may induce flatulence and diarrhoea. While Nigari has currently been replaced by lemon juice in Indonesia, some cooks in China have reported rinsing the Tofu curd up to 7 times before pressing it (Barrett, 1996).

3.3.1. Effect of curd rinsings on isoflavones in Tofu

The impact of curd-rinsing on the isoflavone content of Tofu was then analysed. The results obtained after 7 traditional rinsings (using a Tofu maker) or 3 experimental rinsings (by centrifugation) are presented in Table 1. The table shows that when the rinsing procedure was applied on the already formed curd, its mass was affected, even though this was not statistically significant. However, the isoflavone content could be reduced by nearly 50% using the traditional process, and by up to 66% for the experimental procedure in Pyrex glass-tubes.

3.3.2. Effect of soy-juice pre-cooking on isoflavones in Tofu

The impact of soy-juice cooking duration on the isoflavone content of Tofu is presented in Figs. S3 and S4. The results are also summarized in Table 2. Fig. S3 shows the evolution of isoflavone content in Tofu made from industrial soy-juice, either cooked for 1 min (the recommended cooking time in modern recipes) or cooked for 15 min. This moderate duration already induced a significant decrease in isoflavone (**Gen** + **Daid**) content (52.86 ± 1.40 mg 100 g^{-1} *vs* 42.88 ± 2.41 mg 100 g^{-1} for 1 and 15 min of cooking, respectively).

3.3.3. Effect of soy-bean meal precooking on isoflavones in Tofu

Fig. S4 shows the evolution of isoflavones in Tofu made from soybean-meal. This reproduces the recipes described by Wentland (Wentland, 2013), which are still followed in the Debao-Gangxi region of China. Four cooking durations were tested, from 5 to 60 min. In these cases, the difference was significant after 60 min of cooking. Here, the isoflavone content represents only 30.54% of the isoflavone content of the initial soy-flour.

3.4. Traditional Tempeh preparation

The data obtained during the process included the first rinsing steps, 2 cooking and soaking steps in hot water and, finally, the fermentation steps, which are presented in Fig. 4. The figure shows that with only 34.19 ± 1.82 mg 100 g^{-1} in Tempeh, the percentage of isoflavone remaining was only 18.07% ± 0.96, whereas the initial value in the soybean seeds was 189.25 ± 4.37 mg 100 g^{-1}. Traditional fried Tempeh contained the same amount of isoflavones as traditional raw Tempeh *i.e.* 34.68 ± 2.11 mg 100 g^{-1} isoflavones with 22.43 ± 1.13 mg 100 g^{-1} **Gen** and 12.25 ± 0.98 mg 100 g^{-1} **Daid**. The industrial Tempeh, assayed here for comparison, contained 29.23 ± 1.21 mg 100 g^{-1} of **Gen** and 16.08 ± 0.29 mg 100 g^{-1} **Daid**, totalling 45.32 ± 1.50 mg 100 g^{-1}. The isoflavone content from the initial soybeans used to prepare commercial Tempeh is unknown and therefore the efficiency of the modern preparation on isoflavone removal is unknown.

Table 1
Isoflavone content and % of initial concentrations in Tofu made from traditional or experimental recipes.

Item analysed (n = 3) (mean ± SEM)	Genistein μg mL^{-1} or μg g^{-1}	Daidzein μg mL^{-1} or μg g^{-1}	Gen + Daid μg mL^{-1} or μg g^{-1}	% from initial concentration	Total in sample (mg)	% from initial amount
Traditional preparations						
Initial soy-juice (200 mL)	204.55 ± 6.5	109.8 ± 2.7	314.35 ± 8.6	100%	62.87 ± 18	100%
Tofu from soy-juice cooked 1 min (106 g)	313.10 ± 32.43	156.04 ± 20.11	469.14 ± 49.41	149.24%	49.73 ± 5.24***	79.09%***
Whey (90 mL)	50.01 ± 3.68	43.93 ± 0.49	93.94 ± 4.15	29.88%	8.45 ± 0.46	13.44%
Tofu rinsed 7 times (83.7 g)	268.97 ± 6.58	128.22 ± 5.19	397.19 ± 10.51***	126.35%	33.23 ± 0.88***	52.86%***
Experimental preparations						
Initial soy-juice (5 mL)	206.15 ± 10	111.53 ± 8	317.68 ± 18	100%	1.59 ± 0.09	100%
Tofu control (3.5 g)	253.6 ± 11.56	157.37 ± 8.22	410.98 ± 19.78	129.37%	1.44 ± 0.09	90.57%
Whey control (1.75 mL)	31.73 ± 2.21	38.91 ± 1.58	70.63 ± 3.79	22.23%	0.12 ± 0.02	7.55%
Tofu from ultrafiltrated juice (2.23 g)	72.61 ± 4.47	41.32 ± 1.74	113.93 ± 6.21	35.86%	0.25 ± 0.04***	15.72%***
Whey from Tofu made on ultrafiltrated juice (2.61 mL)	10.66 ± 0.59	10.23 ± 0.31	20.88 ± 0.90	6.57%	0.05 ± 0.004	3.14%
Tofu rinsed 3 times by centrifugation (2.22 g)	168.31 ± 1.04	72.17 ± 3.61	240.48 ± 4.65	75.69%	0.53 ± 0.035***	33.33%***
Whey from Tofu rinsed 3 times by centrifugation (17.5 mL)	18.63 ± 1.30	20.12 ± 1.05	38.75 ± 2.35	12.19%	0.68 ± 0.015	42.77%

*** Significant difference (p < 0.005) compared to control value.

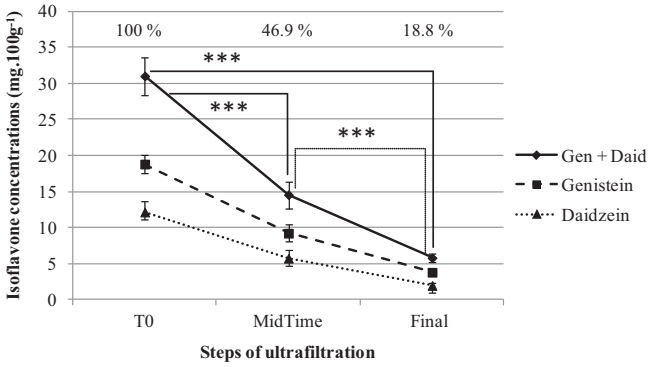

Fig. 3. Evolution of isoflavone content in soy-juice during the selected ultrafiltration process. (T°C 55 °C; P = 400 kPa; Cut-off = 5 KDa; initial pH 8.56; 20 g kg^{-1} $NaHCO_3$). The first value is the isoflavone concentration in initial soy-juice. The second value is obtained after having rinsed the juice with twice the amount of filtrated tap-water. The third value is obtained after the addition of 3 times 10 L of filtrated tap-water. Percentages indicate the isoflavones remaining in the juice at each step of the process. Error bars are SEM.

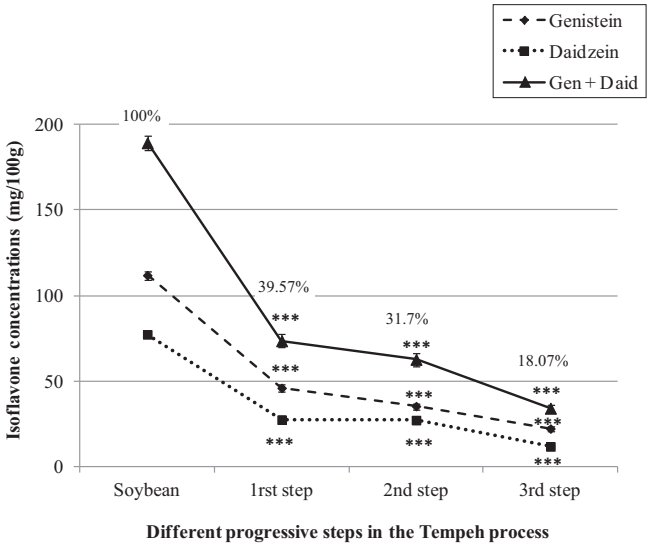

Fig. 4. Evolution of isoflavone concentrations following the traditional steps of Tempeh preparation. Soybeans are the beans used in this cooking experiment. The first step includes 3 rinses of the beans and a 1st cooking (20 min to boiling point + 20 min soaking in hot water). The second step includes 3 rinsings of the precooked beans in tap water and a 2nd cooking step (20 min to boiling point + 20 min soaking in hot water). The third step is fermentation with *Rhizopus oligosporus*. Percentages indicate the isoflavones remaining in the juice at each step of the process. Error bars are SEM. *** indicate a significant difference with $p < 0.005$ compared to initial isoflavone concentration.

4. Discussion

4.1. Justification of the study

Despite on-going controversy about the beneficial or adverse effects of estrogenic isoflavones (Barrett 1996; Bondesson & Gustafsson, 2010), most scientists would probably agree that both effects could be expected from weak estrogens present at the mg range in human and animal foodstuff (Bennetau-Pelissero, 2013). Estrogenic isoflavones are associated with soybean, their main source within the animal and human diet (Table S1). The table combines original data and data from Vergne (Vergne et al., 2008). Isoflavones, with their low estrogenic effects, were considered to be safe, based on the assumption that they had always been consumed at the same amount in Asian countries. However, is modern isoflavone exposure comparable to historical levels in Asian populations? In fact, it has been found that Asian age-old cooking habits empirically eliminated soybean's anti-nutritional factors (Chen, Xu, Zhang, Kong, & Hua, 2014). Domestic soy preparation, including soaking and simmering, lasted several hours (Wentland, 2013). This procedure, effective even in rudimentary conditions, can remove glycosylated isoflavones from soybean because these compounds are water-soluble (Jankowiak, Kantzas, Boom, & van der Goot, 2014). However, as glycosylated isoflavones adsorbed to protein need time to be desorbed (Jankowiak et al., 2014), are modern industrial production methods as efficient at removing phytoestrogens? These considerations raise four further questions: 1) How exactly did traditional Asian cooking habits influence isoflavone content? 2) Is modern-day isoflavone exposure resulting from industrial foodstuff comparable to the age-old Asian diet? 3) If not, and since recent data indicate the potentially adverse effects of isoflavone exposure on reproductive parameters, what type of

Table 2
Summary of isoflavone (daidzein and genistein) concentrations and remaining percentages and percentages per gram of protein in commercial foodstuff and in traditionally cooked or experimental preparations.

N°	Foodstuff	Concentrations mg $100g^{-1}$	% from initial foodstuff	% of protein	mg of isoflavones per gram of protein
Commercial foodstuff					
1	Soy-juice for ultra-filtration*	31.02 ± 2.62	100	4.74	6.54
2	Soy-juice for Tofu preparation	25.6 ± 1.42	100	3.7	6.92
3	Soybean meal	81.7 ± 0.58	100	35.2	2.32
4	Soybean seeds for Tofu and Juice	96.54 ± 0.59	100	36.5	2.64
5	Soybean seeds for Tempeh	189.25 ± 4.37	100	37.2	5.09
6	Soybean flakes	94.67 ± 0.24	100	50	1.89
7	Industrial Tempeh	45.32 ± 1.50	–	21.7	2.09
Experimental and home-made foodstuff					
	Soy-juice after ultra-filtration	5.83 ± 0.62	18.79	4.7	1.24*** (to 1)
	Soy-juice made from dehulled soybean seed (1 h pre-cooking)	2.44 ± 1.12	2.52	3.6	0.68*** (to 4)
	Tofu made from soy-juice (15 min pre-cooking)	42.88 ± 4.82	167.50	12	3.57*** (to 2)
	Tofu made from soybean meal (1 h pre-cooking)	24.95 ± 3.32	30.54	13.8	1.81*** (to 3)
	Soybean flakes (1 h pre-cooking)	13.87 ± 0.15	14.65	45	0.31*** (to 6)
	Pounded soybean flakes (1 h pre-cooking)	10.72 ± 0.32	11.32	42	0.25*** (to 6)
	Home-made Tempeh	34.19 ± 1.82	18.07	21	1.63*** (to 5)

* Mean of 6 different batches collected from February 2014 to September 2014.
*** Significant difference ($p < 0.005$) compared to initial value in the corresponding process.

data is required to improve our knowledge of dietary phytoestrogen health effects? 4) Can we consider isoflavone exposure as homogenous in all soy-consuming countries, irrespective of their degree of industrialisation?

4.2. Discussion of the results

Experimental data about Tofu or soy-juice (Figs. S2, 1, 2 and Table 2) using pounded or un-pounded seeds, showed that prolonged pre-cooking, prior to crushing, eliminated 50–80% of the initial isoflavone content. Present-day manufacturers could use pre-cooking to reduce isoflavone content in soyfood. The longer the soaking and simmering steps before drying solid soyfood, the more complete the elimination of isoflavones (Table 2 and Figs. 1–3). Modern soy-juice, however, being liquid, logically presents the highest isoflavone:protein ratios (Table 2). Tofu curd, prepared from soy-juice, can be rinsed to remove isoflavones at significant rates (35.5–57.3%). Centrifugation of rinsed curd could allow industry to reduce isoflavones. The present experimental ultra-filtration process, which reduced soy-juice isoflavone content by over 80%, confirmed Chinese reports (Jing & Zhang, 2006). It also maintained Tofu protein levels and its transformation ability. Tofu curd formation requires prior elimination of the $NaHCO_3$ added to prevent clotting and membrane clogging. $KHCO_3$ was found to be more efficient than $NaHCO_3$, since only 1.77 g L^{-1} of $KHCO_3$ was necessary. Admittedly, additional work by manufacturers would be needed to ensure that the juice and Tofu obtained via ultra-filtration correspond to consumer taste. Equally, as prolonged cooking and several rinsing steps are not environmentally-friendly, the soy-product industry might not retain them. Although traditional Tempeh preparation effectively removed isoflavones, leaving only 18.07% in the final product, it required 2 L of tap water at each soaking and cooking step. The final water:seed ratio of 16 L:200 g may not, however, be sustainable in industry. Because we do not know the isoflavone levels in the soybean seeds used for industrial Tempeh, no comparison can be made between the modern and traditional processes.

The present study shows that although traditional recipes successfully removed isoflavones, these recipes might be difficult to adapt to industrial soy food production procedures. Therefore, to reduce consumer exposure to isoflavones, various legumes containing vegetable proteins could be used as a replacement. These include pea, lupine and beans (*Vicia faba*), commonly used in manufactured human foodstuff. Variety selection makes their nutritional characteristics much closer to those of soybean, with a much lower isoflavone content (Table S2).

4.3. Consequences of modern processes on consumer exposure to estrogenic isoflavones

The traditional recipes developed here led to low isoflavone levels, thereby confirming earlier findings indicating that soybean curd only contains less than 5% of the soybean isoflavones (Li, Poon, & Woo, 2004). This indicates that historical isoflavone exposure in Asia was lower than the current cumulative exposure of regular soyfood consumers, as confirmed in a study (Liu et al., 2004) of rural Chinese areas pursuing traditional cooking practices. Their isoflavone intake was low (below 15 mg day^{-1}). Current isoflavone intake, however, for an adult in China ranges between 20 and 40 mg day^{-1} (Lee et al., 2014).

When the industrialisation of soybean processing started, knowledge about isoflavones was lacking. This meant that isoflavones were not considered in either traditional or modern industrialised foodstuff. The heating steps were considerably accelerated in industry to reduce energy costs, but fewer isoflavones were removed. Later, when these compounds were discovered in the urine of modern soyfood consumers (Axelson et al., 1982), it was thought that as these probably corresponded to traditional Asian urine concentrations, both modern Asian and Western isoflavone exposures could be considered safe.

Recently, Tofu was reported to be by far the most frequently consumed soyfood in China (Liu et al., 2004). In addition, traditional Tempeh, Tofu, Nato and Miso are solid foodstuffs, all obtained after long simmering or soaking steps (Liu et al., 2004). The subsequent pressing or drying, used to eliminate water, also removed most of the isoflavones. Modern soy-juice, not a traditional drink in Asia (Hirayama et al., 2009; Lee et al., 2014), is made by retaining the cooking water and all its isoflavones, and exhibits the highest isoflavone:protein ratio. Soy-juice was not traditionally consumed in Asia because it is unattractive to Asian populations, who have problems in digesting milk (Vesa, Marteau, & Korpela, 2000). Traditionally, less than 5% of soybean was consumed there as soy-juice (Lee et al., 2014), and this still pertains to present-day Japan (Hirayama et al., 2009). In Western countries, 90% of soyfood is derived from soy-juice. The introduction of soy-juice was followed by that of soy-based infant formulas, which led to an infant isoflavone exposure of 2.3–9.3 mg kg^{-1} day^{-1} (McCarver et al., 2011). These levels far exceed those shown to disrupt the menstrual cycles in premenopausal women (0.75 mg kg^{-1} day^{-1}) (Cassidy, Bingham, & Setchell, 1995).

In Western countries, soybean has become a common part of human diet via soyfood based on soy-juice, and the soy hidden in manufactured food (Table S1). Isoflavones, now the most prevalent and potent estrogenic compounds in human food (Omoruyi et al., 2013), are 1000–10,000 times more concentrated in foodstuff than other xenoestrogens, such as pesticides, themselves 10–100 times less potent than isoflavones (Omoruyi et al., 2013). Consequently, since hidden soy is seldom included in food databases, consumers' isoflavone exposure is generally underestimated.

4.4. Controversial effects of soy isoflavones

Recently, fertility problems have been reported in humans (Lang & Nuevo-Chiquero, 2012; Splingart et al., 2012), and also soy-fed cattle of industrialised countries. The US NTP recently reported toxic effects of **Gen** on rat reproduction at LOAEL of 35 mg kg^{-1} bw day^{-1} (National Toxicology Program, 2008), thereby allowing initial toxic levels in animals and humans to be defined. The exposure of children, adolescents and adults under 50 to significant xenoestrogen levels is thought to impair fertility. Soy isoflavones are now associated with a reduction in sperm count in Asian and US adults (Mumford, Kim, Chen, Boyd, & Buck Louis, 2015 and references included). Over-consumption of soy in women is linked to pituitary, endometrial and menstrual cycle impairments in women (Cassidy et al., 1995; Chandrareddy, Muneyyirci-Delale, McFarlane, & Murad, 2008). Deleterious effects have also been reported in children fed either soy-based formulas (Gilchrist, Moore, Andres, Estroff, & Badger, 2010) or soy in early life (Kim et al., 2011). One study specifically records the effects of soy-based infant formula intake during infancy on adult reproduction, but it does not help in determining whether adult fertility could be affected, as many scientists fear (Badger, Ronis, Hakkak, Rowlands, & Korourian, 2002; McCarver et al., 2011). Reproductive impairments were also reported in domestic animals exposed to phytoestrogens (Bennetts et al., 1946; Yuan et al., 2012). These data suggest that estrogenic isoflavones should be considered, together with other endocrine disruptors, as a potential cause of such fertility problems.

Soy and/or its isoflavones were shown to disrupt the thyroid function (Fruzza, Demeterco-Berggren, & Jones, 2012). Goitres have been observed in hypothyroid babies fed soybean-based infant

formula, and soy isoflavones seem to aggravate any pre-existing hypothyroidism (Sathyapalan et al., 2011).

The controversy about breast cancer is now dying down (Bondesson & Gustafsson, 2010). The current hypotheses are that **Gen** may prevent cancer during its promotion phase via epigenetic effects, thereby protecting Asian women exposed from childhood to modern soyfood (Warri, Saarinen, Makela, & Hilakivi-Clarke, 2008). However, **Gen** also induces the expression of genes involved in breast-cancer cell proliferation in women with estrogen-dependent breast cancer (Shike et al., 2014). Soyfood has proliferative effects on healthy breast cells in premenopausal women (McMichael-Phillips et al., 1998). In addition, **Gen** and **Daid** are growth factors for human estrogen-dependent tumour cells both *in vitro* and in animal models of xenograft nude mice (Du et al., 2012 and references included). In Western menopausal women, the effect of soy on breast cancer is unclear (Bennetau-Pelissero, 2013; Warri et al., 2008). However, most of the existing studies have neglected to include the soy isoflavones hidden in manufactured food, thus reducing the statistical power of their analyses. The Asian diet may, however, be protective for several cancers via other traditional foodstuffs.

Although prostate cancer incidence differs in Western and Asian populations, the occurrence of cancer, as analysed by post-mortem diagnosis, shows similar frequencies between populations (Zlotta & Kuk, 2014). Data show that the tumour estradiol receptor (ER) subtypes are crucial. ERβ-bearing tumours are protected by isoflavones, whereas ERβ2 variant bearing tumours are stimulated (Bennetau-Pelissero, 2013, with references).

The link between colon cancer and soy consumption is unclear. Recent data correlated soy consumption in women with a lower risk of colon cancer (Tse & Eslick, 2014). These results still need more consistent evidence, since soybean may not be the only positive factor involved (Tse & Eslick, 2014).

Meta-analyses also show positive isoflavone effects on the prevention of hot flushes (Bennetau-Pelissero, 2013), and soy extract-based food-supplements are the most popular world-wide for vasomotor menopausal symptoms. The excretion of bone resorption markers is reduced in the peri- and post-menopausal populations by isoflavone intake (60–100 mg day^{-1}) from food supplements (Bennetau-Pelissero, 2013), but this does not prove that isoflavones actually prevent osteoporosis.

Finally, for the FDA, the most consensual effect of soy is a consistent reduction of plasma LDL_{chol} when soybean constitutes a meat substitute. Several studies show that the lowering of plasma LDL_{chol} due to soybean can range from 7 to 10% (Jenkins et al., 2010). However, the relevancy of this biomarker for cardiovascular diseases is currently under debate.

5. Conclusion

Traditional soy preparation in Asia was mostly confined to solid food-stuffs, such as Tofu, Tempeh, Natto, Miso. Soy-juice was, and still is, only occasionally consumed in Asian countries. All of the traditional foodstuffs mentioned above were traditionally prepared after prolonged simmering lasting up to 4 h, or following several rinsing and cooking steps in water. Here we show that simmering in water time-dependently removes isoflavones from the soybean foodstuff. We also show that rinsing and cooking in water allow the glycosilated isoflavones to leak into the water, thereby reducing the isoflavone content in soybeans. As this cooking water is removed from traditional solid soy-based foodstuff, this indicates that the historical exposure to isoflavones was probably low in Asia. Nowadays, in modern Asian countries, the traditional recipes are prepared using industrialised processes, and the rinsing and cooking steps are reduced to save energy and water costs. These new procedures, developed when the effects of isoflavones were largely unknown, retain a high isoflavone:protein ratio in modern soy-based foodstuff. In consequence, the human exposure currently recorded in modern soy-eating countries is most probably higher than in the age-old ones. In addition, in Western countries, where soy-juice and its derived products are mainly consumed, the cooking water is used, together with all of its isoflavones. This explains why soy-juice is the soy-food that exhibits, by far, the highest isoflavone:protein ratio. Here, we showed that precooking soybeans and eliminating the water before crushing them can significantly remove isoflavones from soy-juice, especially if the cooking step that mixes juice and Okara is reduced or omitted. Soy isoflavones are the most potent and prevalent xenoestrogens in the modern consumers' environment. They can aggravate the thyroid status of hypothyroid patients. Equally, the current isoflavone exposure is most probably a recent one. Therefore, soybean should be considered as a modern source of endocrine disruptors, and studied as such.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.126.

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