Isoflavone-enriched Foods: Aspects of Production, Analysis, Sensory Properties and Shelf Life - The PHYTOS Project

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Summary

Epidemiological data suggest that Asian women, consuming traditional soy based foods enjoy better cardiovascular and bone health than their counterparts in Western societies. A large body of data indicate that soy-isoflavones (IF) are involved in protecting from cardiovascular disease, osteoporosis, in reducing the risk of certain cancers and in reducing post-menopausal symptoms. Unlike Asians, Western populations do not consume a lot of soy based foods, resulting in a low intake of IF. Increasing daily IF intake in these populations is either possible by consuming supplements or foods that have been enriched with IF. In the present study we report on various aspects of food enrichment with IF, effects on organoleptic properties and shelf life as well as analysis of IF content in processed foods. The results show that the different processing steps in the preparation of biscuits, including baking, did not affect IF content and the procedure used for bars, in which IF were added to the syrup holding together the cereal flakes, allowed minimal physico-chemical strain to the IF molecules. There were no changes in isoflavonoid concentrations during storage. This study indicates that the production of IF enriched biscuits and cereal based foods is possible and does not present particular technological difficulties. Accordingly, it is possible to supply a western population with IF enriched foods that match their cultural food selections.

1. Introduction

Epidemiological data suggest that Asian women, consuming traditional soy based foods such as tempeh, miso, natto, and fermented soy milk products enjoy better cardiovascular and bone health than their counterparts in Western societies. Such foods are particularly rich in isoflavones (IF), which belong to a class of food components known as phyto-estrogens. The latter are present in a large number of plant-foods, but are most abundant in soybeans\textsuperscript{1-3}. Plant estrogens have a structural and a functional similarity to human estrogens. At present, an increasing number of studies has been published indicating a role of IF may in protecting from cardiovascular disease, osteoporosis, in reducing the risk...
of certain cancers and in reducing post menopausal symptoms\textsuperscript{11-14}. Unlike Asians, in Western populations most of the proteins are obtained from animal source products and carbohydrates along with proteins from grains and roots. These foodstuffs have a low content of IF\textsuperscript{15}. Even vegetarians living in Western countries who consume a wide variety of plant foods may only consume about 10 mg IF/day\textsuperscript{16}. Increasing the intake of plant estrogens in a population that is not historically used to consume a high amount of soy based foods is either possible by consuming supplements or by consuming foods that have been enriched with IF. Evidence that a regular consumption of IF with the daily diet has a protective effect in reducing chronic disease risk in Caucasians has yet to be obtained. One of the preliminary questions in this respect is whether IF, added to a specific food matrix, are not lost during food processing, remain stable during storage and are well absorbed once consumed. Recently, two studies have focussed on the isoflavone content of IF supplements that are on the market in USA\textsuperscript{17} and in Europe\textsuperscript{18}. A careful analysis of the supplement content indicated that there were considerable differences in measured vs. claimed IF content. Additionally, it was observed that the IF concentrates used in the supplements were very different in composition. Soy bean extract containing supplements typically had a high genistein level, whereas supplements derived from the soy germ were high in glycitin and glycinein. Red clover supplements, on the other hand, were high in formononetin and biochanin A, but low in daidzein and genistein. Accordingly, knowledge about these aspects is of great importance for the execution of well-controlled clinical trials that address the effects of isoflavone consumption on health, as in the PHYTOS project.

The overall scientific objective of PHYTOS is to provide scientific evidence about the effects of soy IF (IF) on bone density and metabolism in postmenopausal women living in Europe. The knowledge acquired in this project will help to quantify the biological relevance of physiological IF consumption after menopause in osteoporosis prevention. It will also provide a scientific basis to develop IF-enriched foods specifically designed for this age group. Such foods should be:

- highly palatable (to increase compliance);
- be available in different flavours (to minimise attrition);
- maintain their IF composition and content as well as their organoleptic characteristics over a prolonged period of time.

Isoflavones are known to have a bitter taste when included in food matrix and the degree of bitterness is influenced by the water content of the product. Additionally, it is known that IF have a poor solubility. Both factors are of significance in determining food applications and developing methods to overcome these limitations. Here we report on various aspects of food enrichment with IF, its effect on organoleptic properties, effects shelf life and analysis of IF content in processed foods.

Two IF-enriched food products were developed in this respect: a biscuit and a cereal bar. Data on genistin and daidzein content in food samples were obtained at two intermediate steps of the manufacturing process, in the final product as ready for consumption and after 4, 6 and 9 months of storage. Data on organoleptic properties were obtained at similar moments. Data on how temperature, incubation time, and other manufacturing processes affect the distribution and content of Isoflavones are presented.

2. Manufacture of IF concentrate

The soy isoflavone manufacturing process used soy-solubles obtained from soybeans as the raw material. Soy-
solubles were produced from defatted soybean flakes by solvent extraction (Figure 1).

Soy solubles typically contain 50 to 55% solids, and have a pH of 5.5 to 6.0. These are further processed to obtain a 50% IF concentrate containing 60-75% genistein, 25-35% daidzein, and 1-5% glycine.

3. Food production and sensory testing

3-1. Biscuits

Isoflavones are known to interact with proteins in a way that may potentially decrease their biological activity. In dry biscuits, gluten proteins are added to obtain desired rheological properties of the paste (viscosity, elasticity). Gluten proteins also induce the desired capacity to retain and include gas produced by the raising yeast agents during the dough preparation.

A 3.5% and 1.8% IF content of the paste was used to reach a value of 50 and 100 mg of IF aglycone per biscuit respectively. No significant change in rheological properties of the pastes was observed (viscosity measured by flow, and elasticity measured by compression). After baking it was observed that the isoflavone-enriched biscuits were thinner than standard biscuits (6.1±0.2 mm vs 7.2±0.2 mm), indicating that the addition of IF most probably has resulted in a decrease of the gluten capacity to retain gas during the raising process.

Seven individuals tasted isoflavone enriched biscuits with 50 mg and 100 mg IF. Biscuits containing 100 mg IF were found to be very bitter and none of the test panel subjects would have been willing to consume these biscuits daily during a period of one year. An inclusion level of 50 mg IF/biscuit was found to be acceptable, although a "vegetal after taste" was noted. In order to mask this off taste note, a vanilla aroma (heavy aromatic molecule) was added to the ingredient formula. This led to an overall acceptable product. Biscuits of 8.5 g or 12 g were produced.

3-2. Cereal bars

Cereal bars (30 g) enriched with IF were developed using the manufacture process described in figure 2. The cereal bar matrix is a mixture of binding syrup, cereal ingredient and fruits (flakes, puffed, and fruit). Recipes were adapted in order to maximise the preservation of the isoflavones and to obtain a uniform mix. The binding syrup was cooked, then isoflavones were added and blended and subsequently the addition the cereal ingredients took place. Different tests were carried out to determine the maximum possible concentration of IF in such bars, without a negative impact on taste, resulting in the selection of a bar that contained 50 mg aglycone IF / 30 g bar.

In order to maximise adherence to the study, three flavours were introduced and tested for taste properties. Flavours were pineapple, apricot or grape. The production process was further adapted to maximise the preservation of the IF, and to obtain acceptable organoleptic properties that are expected to be preserved during storage.

The sensory evaluation was done blinded in a triangular test in 15 early postmenopausal women. Participants received three envelopes each containing three cereal bars of the same flavour. On day 1 (Monday), day 4 (Thursday) and day 7 (Sunday) of the testing week, volunteers consumed all 3 cereal bars of the respective envelopes (one in the morning, one at lunch and one in the evening). The test food consumption order was balanced. A questionnaire had to be filled in only when all three bars had been eaten. Nobody else was allowed to eat or to taste the bars to prevent any influence on answers. Questions were asked about overall quality, changes of acceptance during the day, odour, flavour, mouth-feel and willingness to consume again. Open questions about other suggested tastes and texture characteristics were also asked. Fresh
products, as well as products that had been stored for 6 months, were tested for preservation. No differences were observed between the two products, indicating that there will be neither a modification of taste perception nor of texture after 9 months of storage.

Additionally a taste preference test was performed (panel of 15 women). The results obtained showed that the apricot bar was the preferred type, followed by grape and than pineapple. 11 women indicated to be willing to consume 2 bars daily during one year, the remaining 4 subjects said to agree for at least one bar/day.

To evaluate possible geographical differences in fruit flavour preference bars of each flavour were provided to 3 test centres in France, Netherlands and Italy for allowing recruited individuals to taste the product during the recruitment period and define the best personal choice for use during the final study. The preferred flavours were apricot for Italy and France and grape for the Netherlands. Other flavours suggested as desirable by the test participants were chocolate, nuts and hazelnuts, apple, sesame, strawberry, vanilla. Personal remarks were also given to desirably reduce the sugar content/sweetness, to make the bars more crunchy and less sticky and to reduce the size to a format that is easy to consume for a long period of time in addition to the daily meals/diet. As a result of the test, it was decided that pineapple, the less acceptable flavour, would be replaced by the apple flavour, as nut and chocolate would be problematic with respect to lipid content and energy value.

The energy content of the bars after final development was 116 kcal (491 kJ) for apricot and apple and 115 kcal (487 kJ s) for grapes.

Table 1. Content of isoflavones glucosides* in Soy Protein Isoflavone extract

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Total IF (mg/g)</th>
<th>Daidzin (mg/g)</th>
<th>Glycin (mg/g)</th>
<th>Genistin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>461.04</td>
<td>147.06</td>
<td>5.39</td>
<td>305.93</td>
</tr>
<tr>
<td>1B</td>
<td>455.83</td>
<td>145.12</td>
<td>5.43</td>
<td>302.62</td>
</tr>
<tr>
<td>2A</td>
<td>455.83</td>
<td>145.12</td>
<td>5.43</td>
<td>302.62</td>
</tr>
<tr>
<td>2B</td>
<td>465.21</td>
<td>147.95</td>
<td>5.34</td>
<td>309.29</td>
</tr>
<tr>
<td>3A</td>
<td>467.76</td>
<td>150.00</td>
<td>5.63</td>
<td>309.56</td>
</tr>
<tr>
<td>3B</td>
<td>465.99</td>
<td>149.41</td>
<td>5.25</td>
<td>308.61</td>
</tr>
<tr>
<td>Average</td>
<td>461.94</td>
<td>147.44</td>
<td>5.41</td>
<td>306.44</td>
</tr>
</tbody>
</table>

*100 mg IF as glucoside correspond to 60 mg IF as aglycone

subsequently analyzed by HPLC using a C18 column and U.V. detection. A sample was extracted three times and each extract was injected twice. The data obtained, expressed as isoflavone glucosides, are shown in Table 1. In order to minimise the between batch variability the same IF concentrate batch was used throughout the study.

A sample of the Soy Isoflavone Extract was additionally analyzed by the Department of Chemistry of the University of Helsinki (KW) who was in charge of the analysis of the isoflavone content in the test foods as well as in the biological fluids. Various extraction methods were tested and extensive studies using all available techniques (LC-MS, HPLC-coulometry, GC-MS) were performed and to obtain reliable and reproducible results. The isoflavonoid content was analysed as aglycones. Accordingly, for analysis of IF concentrate samples were hydrolysed.

When the IF concentrate was analysed using standard hydrolysis (Helix pomatia enzymes followed by aqueous hydrochloric acid) and extraction procedure (shaking for 4 min with 80% methanol) followed by HPLC coulometric analysis the total isoflavone aglycone content (daidzein and genistein) was 134 mg/g, which was significantly lower than the value obtained by the supplier, 285 mg/g. With a modified extraction procedure it was found that the water phase contained about 8% of isoflavones, which were not extracted in the original procedure. When the IF concentrate was re-analysed using the producer's extraction method followed by HPLC coulometric measurement, the total isoflavone concentration was found to be 167 mg/g. From these analytical experiments it is clear that the hydrolysis and extraction is amenable to losses, ultimately due to the low solubility of free isoflavones, which may explain the difference between

4. Isoflavone content of base material and of test foods

4-1. IF concentrate

The IF extract was analyzed for the content of IF, using high performance liquid chromatography (HPLC). For this procedure, soy protein was extracted with a 80:20 Methanol:water mixture and a filtered aliquot was
methods measuring the conjugates directly as well as after hydrolysis.

In the HPLC coulometric analysis an unknown compound was detected close to the peak of daidzein. This very minor compound did not resolve from daidzein even after as many as 20 HPLC runs. GCMS, LCMS and NMR analysis of an enriched material indicated that it may be an oligosaccharide with glucose and rhamnose moieties. Further studies of the IF concentrate indicated the possible presence of a small quantity of protein and also of lactic acid.

4-2. Foods

Samples of the biscuits and the cereal bars were provided to the university of Helsinki for analysis immediately after production (Time 0), and at different storage times in order to verify the total amount of isoflavones and the possible losses.

Fifty mg sample was weighed and swollen in 0.5 ml of water overnight at room temperature. About 2 ml of EtOH was added and the sample was shaken for 2 min at 2000 rpm at room temperature. The sample was then centrifuged 10 min at 2500 rpm. Supernatant (80% EtOH) was taken into a volumetric flask and filled with 80% EtOH. Samples were diluted with the mobile phase containing 30% eluent B prior to analysis. Analysis was done by HPLC coulometric detection in triplicate and results were confirmed by isotope dilution gas chromatography-mass spectrometry in the selected ion monitoring mode (ID/GC/MS/SIM) using synthesized deuterated internal standards for the correction of losses during the procedure[4].

For cereal bars, the analysis was found to be complicated by the nature of the matrix, being sticky and non-homogeneous. It was found that manual homogenisation did not give reproducible results and that mechanical treatment was required to obtain a fairly homogeneous powder suitable for analysis. The latter was performed as follows: the bar was quenched in liquid nitrogen and gently smashed into small pieces with a hammer. Prior to lyophilisation the samples were milled with a Janke & Kunkel Ika-werk, type A10 crusher under nitrogen atmosphere. Subsequently, after 140 hours of drying the sample was found to be rather grainy. The big grains were then reduced with mortar and pestle. A second milling with the Ika equipment was needed to obtain a homogeneous powder.

4-3. Loss during processing

Isoflavones are assumed to be heat-stable and not to be lost during the dough preparation and subsequent baking process. To check this hypothesis, the IF content of the initial dough and subsequently of the baked biscuit was determined. The quantity measured in the paste before cooking was 2.81 mg/g. During the baking step, water (16%) was evaporated from the biscuit. Correction for this water content resulted in an expected value of 2.81/(1-0.16) = 3.34 mg/g biscuit after the baking process. The analytical result showed a value of 3.17 mg/g. Accordingly, the baking step did not affect the IF level in the final biscuit.

The preparation of cereal bars did not require cooking and no loss was expected in the preparation of such test foods.

4-4. Between batch reproducibility of IF content

The reproducibility of the IF content in the different productions was good, with a CV of less than 10% for biscuits and less than 5% for cereal bars. Part of this variability is also due to the variability of analytical procedures, as indicated by the similar coefficient of variation within batch and between batches for the cereal bars (Table 2).

4-5. Storage losses

A freshly produced IF concentrate sample was tested as well as after 2 years of storage. Data are given in table 3.

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Total IF (mg/g)</th>
<th>Portion size (g)</th>
<th>Total IF per portion (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biscuits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd pilot production</td>
<td>5.27</td>
<td>8.5</td>
<td>44.8</td>
</tr>
<tr>
<td>2nd production</td>
<td>4.44</td>
<td>12</td>
<td>53.3</td>
</tr>
<tr>
<td>3rd production</td>
<td>4.97</td>
<td>12</td>
<td>59.6</td>
</tr>
<tr>
<td>Bars</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Production(sample 1)</td>
<td>2.31</td>
<td>30</td>
<td>69.3</td>
</tr>
<tr>
<td>1st Production(sample 2)</td>
<td>2.43</td>
<td>30</td>
<td>72.9</td>
</tr>
<tr>
<td>2nd Production(sample 1)</td>
<td>2.16</td>
<td>30</td>
<td>64.8</td>
</tr>
<tr>
<td>2nd Production(sample 2)</td>
<td>2.35</td>
<td>30</td>
<td>70.5</td>
</tr>
</tbody>
</table>
Table 3. Effects of storage on IF content of concentrate

<table>
<thead>
<tr>
<th></th>
<th>Daidzin (mg/g)</th>
<th>Genistin (mg/g)</th>
<th>Daidzein (mg/g)</th>
<th>Genistein (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At production</td>
<td>147.4</td>
<td>306.4</td>
<td>1.9</td>
<td>0.8</td>
</tr>
<tr>
<td>2 year storage</td>
<td>150.0</td>
<td>254.0</td>
<td>1.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Figure 3. Total IF content (as aglycones) in biscuits at different storage times

Table 4. Total aglycone IF content of cereal bars at different storage times

<table>
<thead>
<tr>
<th>Time</th>
<th>Total IF (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.54</td>
</tr>
<tr>
<td>4 months</td>
<td>1.38</td>
</tr>
</tbody>
</table>

and show a small loss (15%) of the glucoside genistin only.

Food samples were stored in their own original packages (unopened) at room temperature in the dark. Repeated analyses of IF content in biscuits indicated that losses of total IF were negligible, up to one year (Figure 3). Effects of storage were also studied in cereal bars, but only up to 4 months. The assay also shows that losses are negligible. The differences in the mean IF content in bars reported in Table 2 and 4 are due to the homogenisation and extraction technique. The storage test was in fact performed on bars homogenised by an ordinary mixer.

5. Discussion and conclusions

The technological component of the PHYTOS project has allowed the development of IF-enriched foods specifically designed for post-menopausal women, who could be used in a clinical trial, but could also be considered for further commercial exploitation. The requirement for such foods was that they should be palatable, so as to allow maximum compliance, and maintain their IF content throughout the shelf life of the products.

The main constraint of the IF enrichment is the bitter or metallic after taste. This was not a big issue and the cereal matrix, with the addition of fruit flavours, allowed to reach relatively high concentrations in small portions, as a 12 g biscuit or a 30 g cereal bar. This allows a convenient introduction of IF enriched food in the diet without major rearrangements that the use of soy based products might require. Biscuits can be used in the context of different meals or as small snack itself, and are common items in the diet of most women in Europe, and there was no problem in their acceptance. Cereal bars are mainly stand-alone snacks and not always popular in different women’s populations. The use of different flavours allowed achieving good acceptability.

The different processing steps in the preparation of biscuits, including baking, did not affect IF content and the procedure used for bars, in which IF were added to the syrup holding together the cereal flakes, allowed minimal physico-chemical strain to the IF molecules. Such procedures also allowed a relatively constant concentration of IF in the different production batches. This is an important issue if foods need to be used to achieve a constant intake of bioactive compounds.

There were no changes in isoflavonoid concentrations during storage. The isoflavone skeleton is a rather stable chemical structure and the assays repeated at different storage times proved that IF concentration was maintained in the two food matrices used. The quality control of the foods required the adaptation of analytical techniques, in consideration of the high affinity of IF for sugars.

This study indicates that the production of IF enriched cereal based foods is possible and does not present particular technological difficulties. If efficacy trials indicate that soy IF intake is useful to protect from the development of chronic diseases associated to lower estrogen levels, than the use of IF enriched food is an option that should be considered for its convenience and acceptability.
【日本語訳（要旨）】
イソフラボン強化食品：製造、分析、官能特性、保存安定性の観点から一PHYTOSプロジェクト
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疫学上のデータによれば、伝統的に大豆ベース食品を摂取しているアジアの女性は、西洋社会の女性に比べて、心臓血管や骨の健康状態の良いことが示されている。大豆イソフラボン（IF）が、心臓血管病や骨粗鬆症の発症を防止し、ある種の癌や閉経後骨粗鬆症の危険性を減少させるというデータは非常に多い。アジアの女性に比し、西洋女性は大豆ベース食品をあまり摂取しないので、IF摂取量が低く、西洋女性の日々のIF摂取量を高めるとは、IF強化食品の摂取で実現できる。本研究で、IF強化食品の官能特性、保存安定性に関する効果、加工食品中のIF含量の分析等、多面的に検討したことを報告する。その結果、パスタやピザを調製する段階でもIF含有には変化が認められず、シリアルフレークと混合させたシロップにIFを加えたバーーでは、IF分子に及ぼす物理的、化学的変化はほとんど認められなかった。保存中のイソフラボノイド濃度に変化は認められなかった。本研究で、IFを強化したピザやシリアル食品を作ること、特別な技術的問題のないことが明らかになった。さらに、西洋社会の女性に対し、食文化にあったIF強化食品を提供することが可能である。
PROFILE

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Dr Brouns obtained a PhD in Nutritional Physiology at the Maastricht University in the Netherlands on the Topic "Food and Fluid Related Aspects in Highly Trained athletes". For this work he was awarded with the Netherlands Sports Medicine Award.

He has 25 years experience in the field of life sciences and health nutrition. He joined Wander Dietetics in the Netherlands in 1980 as head of the Department of Nutritional Sciences and Consumer Information. Later, in 1988 he joined Sandoz Nutrition Ltd. in Switzerland in the function of Nutrition Research Manager, where he developed the Isostar Sports Nutrition concepts and initiated/chaired the Isostar Sports Nutrition Foundation from 1992 until 1999. From 1992-1999 he was head of the Novartis Nutrition Research Unit with a focus on functional foods research in particular related to heart health, overweight/diabetes, mental performance, bone health and sports nutrition.

He joined Erdarla Behin-Say and Cerestar in 1999, for the Global Health & Nutrition Group. Today he is Research Fellow and Manager Nutritional Sciences Europe at the Cargill R&D Center, Vilvoorde, Belgium. He has a guest research position at the Nutrition and Toxicology Research Institute, Dep. of Human Biology, Maastricht University, The Netherlands. His current research interests focus especially aspects of obesity, diabetes and gut health. He has published extensively, both scientifically and in popular journals and books and is a global speaker in the field of Life Sciences and Nutrition. He fulfilled various chairmanships in industrial and academic organizations such as ILSI Europe, Brussels and IDACE, París. He obtained fellow ships for the American College of Sports Medicine and the European College of Sports Sciences and is an invited active member of the British Nutrition Society. He is a registered Biomedical Researcher Nutritional Sciences of the Dutch Academy of Food Science.

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