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Influence of the dehydration process on active compounds of okara during its fractionation

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Abstract: Okara (residue of the soymilk manufacture) is rich in proteins, fibres and lipids. It also contains isoflavones that possess health-promoting properties. A new method has been developed for the valorization of fibres from okara by hydrolysis of insoluble proteins with a protease and removal of the oil. Three different processes were investigated: the first one involved delipidation and drying prior to proteolysis and led to the highest content of fibre (80%) in the final product. The second used proteolysis on crude okara followed by solvent delipidation–dehydration and gave an intermediate content of fibre (75%). The last process was totally enzymatic (proteolysis and lipolysis) and gave the lowest content of fibre in the final product (50%). Fibre water-holding capacity was correlated to the total dietary fibre content of each sample. It was preferable to use crude okara for hydrolysis, since oven-drying during the process decreased the water-holding capacity and modified the isoflavone profile of okara.

Keywords: dietary fibres; okara valorization; enzymatic process; water-holding capacity; isoflavone

INTRODUCTION

Okara is the insoluble residue of the production of soymilk from soybean seeds. This by-product is very fermentable as its moisture content is high (75–85% of water).[1] Therefore its industrial use is not common and it is considered a by-product. Okara is quite rich in proteins (20–30%), lipids (9–20%) and carbohydrates (more than 50%), especially fibres (9–20%, ie 96% of the soybean fibres).[1,2] The composition of fibres, has been described as 12% hemicellulose, 5.6% cellulose, 12% lignin and 0.16% phytic acids.[1] Furthermore, okara contains isoflavonoids[3,4] which are thought to have a positive effect on health (ie lowering of certain hormone-dependent cancers, cardiovascular disease and osteoporosis), possibly due to their antioxidative properties[5,6] and their ability to bind the oestrogen receptor.

Valorization of Okara may be achieved by fractionation of each component. Soluble polysaccharides have been extracted by Maeda,[7] Yamaguchi *et al*,[8] Yoshii *et al*[9] and Kato and Konishi.[10] Proteins have also been purified by Ma *et al*[11] and Chan and Ma[12] on the basis of basic, acid or trypsic hydrolysis. Okara could be used to increase the fibre content in cereal products,[4] but there is little information available about the extraction of okara fibres. Soya fibres are supposed to have good nutritional and functional properties.[13,14] The neutral taste and the absence of colour of such fibres makes them suitable for incorporation into food products without any change in their quality, unlike those from wheat fibres.[15] In the same way, fibre products derived from legume cotyledons have major advantages over cereal bran, including their white colour and the neutrality of flavour.[16]

In this study, we have focused on the extraction of total dietary fibres from okara, and particularly on a preliminary dehydration process. We choose the water-holding capacity (WHC) as a good parameter to relate to the mechanical effect of fibres on gastrointestinal function (increased faecal weight, decreased transit time, etc).[17] In addition, the evolution of isoflavones has been considered.

MATERIALS AND METHODS

Materials

Okara was obtained from Nutrition et Soja (Revel, France). It was the insoluble by-product in the manufacture of soybean milk, obtained from dehulled soybean seeds. After a start-draining step, okara was frozen to avoid any fermentation. Information from the supplier was that the size of the majority of the particles was between 0.5 and 1 mm.

The enzymes used were: protease P-5380 (Subtilisin Carlsberg from *Bacillus licheniformis*) (Sigma); Alcalase 2.4 L (Subtilisin Carlsberg from *Bacillus licheniformis*)

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(Novo Nordisk); Neutrase 0.5 L (*Bacillus amyloliquefaciens*) (Novo Nordisk); Lipase lipolyve R from *Rhyzopus Oryzae* (Lyven, Paris, France)

The Buffers used were: for Neutrase: 0.05 M phosphate, pH 6.0; for Alcalase and protease P-5380: 0.09 M phosphate, pH 7.5; for lipase: 0.05 M phosphate pH 7.0.

Composition of the products (okara and fibre samples)

Dry matter

Okara was dried in a ventilated incubator at 80 °C for 16 h (Heraeus Instruments UT6120, Haufer, Germany) to constant weight. Dried okara was crushed before the determination of its dry matter content. The average coefficient of variation (CV) for these determinations was 1.3%.

Lipids

Fat was removed from dried okara using Soxhlet extraction with hexane (200 ml for a 3 g sample at 60 °C for 4 h), or by stirring dried okara in acetone (100 ml for 3 g of dried okara, at room temperature for 1 h before filtration with Durieux filter No 111). The average CV for these determinations was 11%.

Proteins

Analysis of proteins was performed by Dumas nitrogen determination (NA 2000 of Fisons Instruments, Courtabeuf, France) using N × 6.25 as conversion and the CV for these determinations was 2.6%.

Free sugars

Free sugars were extracted in hot 80% ethanol (1 g of defatted dried okara with 100 ml of 80% ethanol at 60 °C for 30 min) and the residue was oven-dried for 16 h at 80 °C before being weighed. Average CV for these determinations was 5.9%.

Total dietary fibre (TDF)

The determination of the dietary fibre content of the starting materials was done according to Prosky *et al*.[18] For samples enriched in fibres (after the enzymatic process), we used the simple procedure validated by Li and Cardozo[19] which is adapted for non-starchy materials. The average CV for these determinations was 8%.

Ash

Organic material was incinerated in a Muffle furnace (Niberthem Model L9:L6) (Niberthem, Lilienthal, Germany) at 550 °C for 5 h[20] and the residual mass determined. The average CV for these determinations was 2.5%.

Analysis of isoflavones

Extraction of isoflavones was performed with 0.1 g of freeze-dried sample in 80% methanol. The sample was stirred rapidly (vortex IKA MS 1) (IKA, Staufen, Germany) for 2 min and left for 2 h in a rotating stirrer. The suspension was then centrifuged at 24 000 × *g* for 20 min (Jouan MR 22 i) (Jouan, Winchester, USA). The supernatant was filtered (0.45 µm) before HPLC analysis. The HPLC analysis was based on Elridge's[21] method using a C8 column (Cil Cluzeau, Sainte Foy la Grande, France) with a Spectrasystem (Finnigan, Courtabeuf, France) HPLC system, using water acidified with 0.1% TFA and acetonitrile as eluant. The average CV of the method was less than 5 %. Each experiment was done twice. ANOVA was performed on the final data, the residual mean square being considered as the standard deviation to maximize the error on each set of data.

Enzymatic proteolysis of okara

Crude okara (4 g) or oil-extracted dried okara (1 g) was stirred with 60 ml of phosphate buffer at specific pH values (pH 6 for Neutrase and 7.5 for Alcalase and protease P-5380). When the mix was at the required temperature (50 °C for neutrase and 60 °C for alcalase), protease was added (Neutrase or Alcalase: 40–150 µl, P-5380 protease: 5 mg) and the mixture was incubated for 3–480 min. Reaction was stopped by plunging the beaker into a cold water-bath until room temperature was attained. In the case of crude okara, two different processes were used for delipidation.

Delipidation with solvent

The pH was adjusted to 4.5 with 1 M HCl before adding four times the residual volume of 94% ethanol preheated to 60 °C. After continuous agitation until room temperature had been reached, the sample was left at rest for 30 min and filtered through Durieux filter No 111. The residue was washed three times with 20 ml of 78% EtOH, two times with 10 ml of 94% EtOH, and two times with 10 ml of acetone (to dry). The latter procedure led to complete removal of lipids (no lipid residue after Soxhlet extraction). In the case of crude okara, 50 ml of hot acetone (60 °C) was added to the residue and the mixture was stirred for 30 min at room temperature before filtration and washing of the oil-extracted residue. The fibre residue was oven-dried for 30 min (80 °C) and weighed.

Lipolysis

After the end of proteolysis with Alcalase, lipolysis was performed with Lipolive R at 40 °C in a 0.05 M phosphate buffer. The pH was increased to pH 7.0, which is the optimum pH for this enzyme. The hydrolysis was performed for 20 min and the quantity of enzyme used was 5 mg.

Degree of proteolysis

The spectrophotometric method uses the reaction of trinitrobenzenesulfonic acid (TNBS) with primary amines produced by cleavage of the peptide bonds. The 100% level was the total hydrolysis with Alcalase

(500 µl with 2 g of dried okara during 22 h).[22] The CV of the method was between 5 and 5.5%.

Water-holding capacity (WHC)

We have adapted the Quinn and Paton process.[23]

Estimation of the water-holding capacity

Distilled H_2O (3.5 ml) was added to a 100-mg (Ws) sample of material in a centrifuge tube. The mixture was vortexed for 2 min, left to rest for 30 min and then it was centrifuged (20 °C, 18 000 × *g*, 10 min) (Jouan MR 22i) (Jouan, Winchester, USA). Supernatant was removed, the centrifuged tubes were turned upside down for 30 min and weighed (Wh + tube).

The estimated WHC was recorded as the difference between the weight of the hydrated sample (Wh) and the original product (Ws) (g) / weight of the dry product (Ws) (g).

Adjusted water-holding capacity (by addition of a minimal quantity of water)

One hundred milligrams (Wf) of the same fibre sample was weighed into a centrifuged tube. Subsequently [0.25 + (estimated WHC × Wf)] ml of distilled H_2O was added, and the mixture was vigorously stirred with a spatula. After 2 h, the tube was centrifuged and turned round for 30 min to remove the supernatant. Adjusted WHC was calculated as described above. The CV of both methods ranged from 5 to 13%, depending on the fibre content.

RESULTS AND DISCUSSION

Composition of the starting materials

The water content of okara used in this study was 68.91%, which is lower than results obtained by Rinaldi *et al*[4] (77.65%) or Bourne *et al*[24] (76.8%). The wetness of the product is dependent both on the efficiency of the decantation process used in the process of soymilk and on the ability of seed components to absorb water.[25] The high water content made this product fermentable and its industrial valorization difficult. Okara was frozen (−18 °C) or oven-dried (100 °C) to avoid its degradation. Thus, studies were made later directly with the two materials, 'crude (frozen) okara' or 'dried okara'.

Okara composition depends either on the soymilk preparation process (especially the grinding step) or on the quality of the raw material (seed variety and quality). The composition of dried okara (Table 1) was comparable with the composition of okara described by Schweizer *et al*,[26] with a high content of both protein (37.5%) and total dietary fibre (31.1%). The latter figure was intermediate between those of Van der Riet *et al*[27] (52–58%) and of Bourne *et al*[24] (14.5% of fibres, probably for soluble fibres). Fat represented the 20% of the dry matter, which is higher than results obtained by Bourne *et al*[24] (15.2%), or Van der Riet *et al*[27] (9.3–10.9%). Thus the variation in composition reported by authors underlines the variation in the process or in the raw material. Some authors reported additional compositional details about phytic acids (0.16–1.2%),[27,28] hemicellulose (12%),[28] cellulose (5.6%)[27] and lignin (11.7%).[28]

The amounts of isoflavones identified in okara are presented in Table 2. The results are comparable with those obtained by Rinaldi *et al*[4] but were higher than those of Jackson *et al*[29] and of Wang and Murphy.[30] The latter authors used a laboratory process for soymilk preparation which was quite different from the industrial one used by our laboratory and that of Rinaldi *et al*.[4]

The quantity of isoflavones encountered in okara represents almost 30% of the isoflavones in soybean.[29] The five common isoflavones usually identified in soybean were present in okara: daidzin, genistin and their malonyl ester forms accounted for 96% of the total isoflavones. This was in agreement with Wang and Murphy[31] for soybean or soybean flour. In our sample the malonyl genistin was the major isoflavone (more than 50%) which is in agreement with Rinaldi *et al*.[4]

Transformation of the materials by enzymatic process

The method of transformation of okara followed the same general enzymatic process (Fig 1). Three different processes were investigated. The first one involved delipidation and drying prior to proteolysis, which led to the higher content of fibre in the

Table 1. Composition of okara[a] (mean ± SD of three replications)

	Composition (g Kg^{-1} dry matter)
Protein	375 ± 5
Fat	206 ± 12
Sugars	66 ± 2
Starch	1.8 ± 0.2
Ash	38.7 ± 0.5
Total dietary fibres	318 ± 13
Dry matter	311 ± 2 (g Kg^{-1} material)

[a] Okara is a soya pulp obtained as a by product in the soymilk manufacture (Nutrition et Soja, Revel, France)

Table 2. Isoflavones identified in okara (mg g^{-1} of dry matter)

Component	Okara A (µg g^{-1})[a]	Okara B (µg g^{-1})[b]
Daidzin	118	108
Genistin	200	141
Malonyl daidzin	308	399
Malonyl genistin	712	489
Acetyl daidzin	60	0
Acetyl genistin	0	80
Genistein	0	24
Total	1398	1241

[a] Okara analyzed in this study by method of Elridge;[21] the CV was <5%.

[b] Okara analyzed by Rinaldi *et al*.[4]

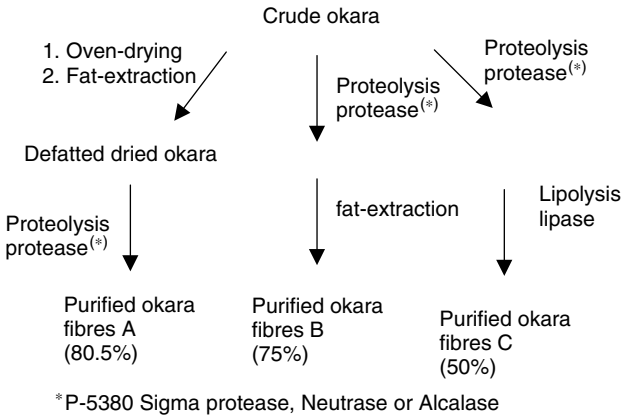

Figure 1. Scheme of purification for okara fibres.

final product. The second used proteolysis on crude okara followed by solvent delipidation–dehydration and gave intermediate content of fibre. The third process was totally enzymatic and gave the lowest contents of fibre in the final product.

Composition of the purified okara fibres

Table 3 presents the fibres composition after the enzymatic purification process: the TDF level in okara could be increased to 80.5% (samples 1–5) and 75% (samples 6 and 7). This was comparable with the TDF content of 'Fibrim' (a commercial soybean fibre sample) (75%),[14] or other 'commercial soy polysaccharides' (77.6%).[15]

The WHC of okara fibres were in agreements with commercial fibres checked in our laboratory in the same way (ie pea fibres with WHC ranging from 7.8 to 16.8 and TDF ranging from 50 to 82).[32]

Okara fibre samples were resistant to a complete hydrolysis of the residual proteins, especially for okara fibre B from crude okara. According to Yoshii *et al*,[9] this is due to special regions called 'non-egg-box regions' in which there is an association between proteins and fibres. Furthermore, Chan and Ma[12,33] insist that okara protein, has a low solubility despite enzymic or acidic hydrolysis, which underlines the difficulty for protein removal.

It was also remarkable that the lipase did not lead to an increase in the fibre content in the product: the residual content of lipid using the lipase was still 14%, which had a negative effect on TDF (46%). This could be due to other interactions between lipids and proteins and to the low solubility of lipids in water. In contrast, delipidation by organic solvents, led to a negligible content of lipids in the final product (Table 3). The latter method for lipid removal was therefore selected.

Optimization of the proteolysis and fibre extraction of okara

The first proteolysis attempts were made from defatted dried okara, with P-5380 Sigma protease (Subtilisin Carlsberg). This protease has been used by Prosky *et al*[18] in AOAC enzymatic-gravimetric method of total dietary fibre analysis in foods. Subsequently, industrial proteases were used, as Neutrase and Alcalase such as from Novo Nordisk. Alcalase (of which Subtilisin Carlsberg is the main enzyme component) led to the best yield of fibres (80.5%). This endopeptidase was used for the optimization of the proteolysis because it was relevant to a process at an industrial scale.

According to Novo Nordisk, Alcalase must be used at the proper pH and temperature: the optimal conditions for this endopeptidase are temperatures about 60 °C and pH values between 6.5 and 8.5. These conditions were tested on okara. In this case, pH had to be controlled regularly: at low concentration of substrate (1 g of defatted dried okara in 50 ml of buffer), 0.09 M phosphate buffer was sufficient to maintain pH between 6.5 and 7.5. At a higher concentration of okara, it was necessary to add 0.1 or 1 M NaOH to maintain pH values up to 7. Acidification of the solution during proteolysis is well known. Titration can be used to follow the proteolytic process and estimate the degree of hydrolysis (DH) for different protein raw materials.[22] Keeping Alcalase activity is a function of the nature and concentration of proteins present. Thus, Alcalase is susceptible to degradation during the reaction, despite precise control of temperature and pH. With the purpose of maintaining an optimal enzymatic activity throughout the reaction time, we added the same amount of Alcalase (150 µl) in three steps (3×50 µl) during 90 min. It did not seem to have any effects on the yield of total dietary fibre.

It was interesting to consider proteolysis directly on crude, non-dried okara, because reaction occurred in aqueous medium. However, we did not know the influence of fat on the reaction. Prosky *et al*[18] recommend fat-extraction prior to TDF analysis of samples if they contain more than 5% fat. Proteolysis of crude okara led to a sample in which the content of fat was higher than the 30% in the initial fat. So an additional step of oil-extraction was introduced at the end of the process to obtain a sample of okara fibres that were entirely defatted, and with sufficient total dietary fibre content (75%).

Higher and lower concentrations of Alcalase were tested on crude or defatted dried okara. We found that 75–100 µl was an appropriate amount of enzyme to hydrolysis 1 g of defatted dried okara or 4.16 g of crude okara, with a fibre yield of about 75%. No increase in the yield was observed for an increase of protease concentration higher than 150 µl Alcalase g^{-1} of defatted dried okara.

The evolution of TDF yield with proteolysis time and the amount of Alcalase was very good (Fig 2). Reaction was stopped at different proteolysis times and for different Alcalase concentrations. The yield of TDF in purified samples increased regularly with time of proteolysis. The reaction occurred faster for higher concentrations of Alcalase. Fibre enrichment was more important at the beginning of the proteolysis

Table 3. Hydrolysis of okara and composition or purified product

Sample	Enzyme	Conditions of proteolysis	Isolated product			
			Fat ($mg\,Kg^{-1}$)	Proteins ($mg\,Kg^{-1}$)	Ash ($mg\,Kg^{-1}$)	TDF ($mg\,Kg^{-1}$)
1 Defatted dried okara (1 g)	P-5380 (Sigma), 6 mg	pH = 7.5; 60 °C; 3H[a]	0 g Kg^{-1}	160 g Kg^{-1}	80 g Kg^{-1}	760 g Kg^{-1}
2 Defatted dried okara (1 g)	Neutrase (Novo), 600 µl[b]	pH = 6; 50 °C; 4H15[c]	0 g Kg^{-1}	332 g Kg^{-1}	78 g Kg^{-1}	590 g Kg^{-1}
3 Defatted dried okara (1 g)	Alcalase (Novo), 100 µl	pH = 7.5; 60 °C; 4H30[c]	0 g Kg^{-1}	121 g Kg^{-1}	74 g Kg^{-1}	805 g Kg^{-1}
4 Defatted dried okara (1 g)	Alcalase (Novo), 3 × 50 µl	pH = 7.3; 60 °C; 4H30[c]	0 g Kg^{-1}	144 g Kg^{-1}	68 g Kg^{-1}	788 g Kg^{-1}
5 Defatted dried okara (1 g)	Alcalase (Novo), 150 µl	pH = 7.29; 60 °C; 4H30[c]	0 g Kg^{-1}	113 g Kg^{-1}	69 g Kg^{-1}	818 g Kg^{-1}
6 Crude okara (6 g)	Alcalase (Novo), 100 µl	pH = 7.5; 60 °C; 4H30[c]	137 g Kg^{-1}	180 g Kg^{-1}	90 g Kg^{-1}	593 g Kg^{-1}
7 Crude okara (4.2 g)	Alcalase (Novo), 100 µl	pH = 7.5; 60 °C; 4H30, defattening after proteolysis	0 g Kg^{-1}	175 g Kg^{-1}	70 g Kg^{-1}	755 g Kg^{-1}

[a] Adaptation of the Prosky's procedure[18] for determination of TDF.
[b] We used more Neutrase whose concentration is weaker than Alcalase (0.5AU g^{-1} for Neutrase and 2.4AU g^{-1} for Alcalase).
[c] Use of Li and Cardozo's method[19] for non-starchy material.

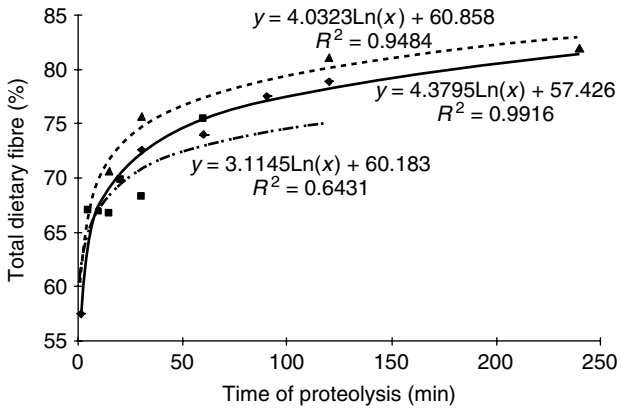

Figure 2. Evolution of total dietary fibre amount in the okara fibre product with proteolysis time and amount of Alcalase: (■) 40 µl Alcalase; (⧫) 75 µl Alcalase; (▲) 150 µl Alcalase (substrate was 3.2 g of crude okara).

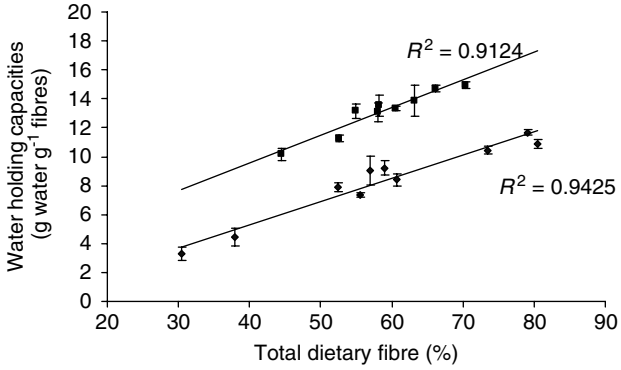

Figure 3. Evolution of water-holding capacity with TDF level: dried and crude okara. (⧫) Fibres obtained from proteolysis of dried okara with different proteases (Alcalase and Neutrase); (■) fibres obtained from proteolysis of crude okara.

reaction which was underlined by the exponential appearance of the curves. This was in agreement with the following observations if we stabilize the pH by adding NaOH: during the first 30 min, we had to add NaOH regularly to maintain pH values up to 7; after 1 h, the pH could be maintained without any addition of NaOH. Adler-Nissen's method[34] (measurement of primary amines produced during proteolysis) was used to follow the enzymatic reaction more precisely. We obtained a curve with an exponential appearance which was typical for enzymatic reactions. The curve rapidly reached a plateau at 50% of the maximum of hydrolysis, indicating that 30 min was a good length of time for the reaction.

Influence of drying on the byproduct

Influence on the water-holding capacity of okara fibres

Functional properties of food fibre ingredients are associated with their (WHCs).[17] Okara fibre samples obtained in this study by complete or partial hydrolysis of proteins were tested for their WHC, and the results were correlated to the TDF level (Fig 3).

Fibres obtained from dried okara presented good water-holding capacities. The proteolysis of crude

non-dried okara produced fibre samples with higher WHC values at comparable TDF content. Unlike drying using organic solvents, the oven drying of crude okara seemed to alter fibre structure and modified WHC (lower WHC values of fibres obtained from oven-dried okara). This hypothesis was supported by a study of Auffret *et al*[35] about functional properties of sugar beet fibres: drying at 110 °C produced retraction of fibres, which does not occur with chemical drying (ethanol or acetone). Furthermore Rinaldi *et al*[4] have reported an increase of insoluble fibre after an extrusion treatment of a mix of okara and flour. Oven-drying must reduce the fibres' capacity to rehydrate. This hypothesis was checked by drying okara fibers obtained from crude (non-dried) okara at 80 °C. Lower WHC values were obtained after drying (nearer the regression curve obtained from oven-dried okara: WHC = 13 for TDF = 76.)

Influence of drying on isoflavones

There was no drastic decrease of the total content of isoflavones from the heat treatment of okara. The concentrations were around 140 $\mu g\, g^{-1}$ with no statistical difference. Several authors have noticed a decrease of isoflavone content due to heat treatments,[4,29,30] but they used hydrated samples whereas our heat treatment was performed on freeze-dried products. The influence of water is also underlined by the influence of soaking without any heat treatment.[29,30]

The effect of heat treatment on isoflavones forms is presented Fig 4. Some modification of the different forms of isoflavones has been observed. During processing, the detectable levels of aglycones, glucosides and acetyl glycoside groups increased, whilst the corresponding malonyl glucosides decreased. These kinds of results have also been pointed out after heat treatment due to extrusion[4] or during the manufacture of soymilk.[29] An hypothesis of Wang and Murphy[31] suggests a decarboxylation between malonyl and acetyl, hydrolysis between acetyl and glucoside, and glycolysis between glucoside and aglucone forms. In our case, we could suppose that, at 85 °C, only decarboxylation and hydrolysis occurred, whereas at 110 °C glycolysis also occurred. Thus heat treatments in dry conditions do not alter the content of isoflavones but induce a qualitative change in their forms.

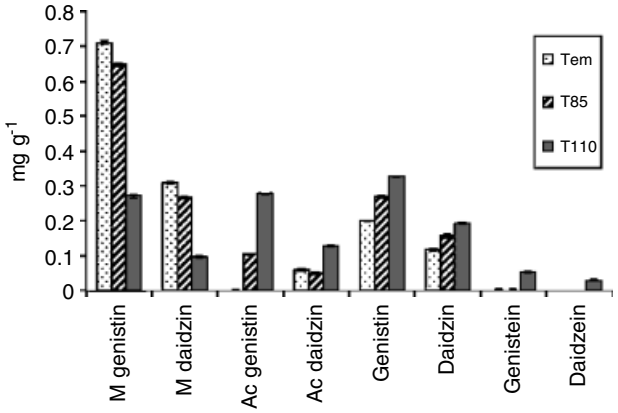

Figure 4. Evolution of the type of isoflavonoids in okara according to heat treatment tem: freeze drying, T85 or T100: drying in an incubator at 85 °C or 110 °C respectively. M = malonyl, Ac = acetyl. Each experiment has been done twice. Anova has been performed on the final data. The residual mean square has been considered as the standard deviation to maximize the error on each data set.

In conclusion, we advise that fibres should not be dried at high temperatures, which can alter the microstructure of fibres and decrease WHC as well as produce a change in the different forms of isoflavones.

CONCLUSION

Okara may be a good and original source for total dietary fibres which enables another valorization of this by-product generally considered as a waste. The present method could be developed on an industrial scale but suffers from the use of solvent for the removal of the residual fat. The enzymatic process for delipidation presented in this study did not reveal an actual capacity for fibre purification. Nevertheless, since the composition of okara is quite variable, it is maybe possible to find a by-product with a low fat content which can lead to a small amounts of lipids in the fibres. The fibres have a quite an interesting WHC and could be used in food products. Nevertheless, the amount of antinutritional factors has to be considered before using such fibres as ingredients.

Attention should be paid to the processing of the okara as raw material. This has to be controlled carefully in order to prevent denaturation of fibres and modification of the isoflavones profile, which could be a drawback for the further valorisation of okara.

Generally speaking, the use of enzymes to remove proteins (or starch) could be extended to other sources of fibre products for the purpose of having dietary fibre ingredients with various technological and nutritional properties.

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