

Separation of isoflavones from okara

Process mechanisms & synthesis

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Chapter 1

Introduction

BY-PRODUCT UTILISATION IN THE FOOD INDUSTRY

Food production requires many resources. One of the main causes is the incomplete use of raw materials and waste generation during production and consumption [1]. Reduction of food wastage and better use of resources would help to meet the demand of 60% more food production that is estimated to be needed for the world population in 2050. Global food wastage furthermore contributes significantly to environmental consequences because of energy and resource usage and associated greenhouse gas emissions that can be avoided [2].

Within the food value chain in developed countries, the second largest losses (39%) of food occur during food manufacturing, following the 42% of food wastes that are produced by the end consumer in households [3]. For both environmental and economic reasons, the large amount of side streams produced during manufacturing of food products has been investigated for the recovery of valuable components, its potential for recycling or for upgrading [4].

Important examples of the successful recovery strategies of ingredients from by-products with commercial application are the production of natural sweeteners from citrus fruits, or the production of whey protein isolates as food supplements from cheese whey [5]. However, the exploitation of industrial by-products goes far beyond that, and an increasingly large number of by-products and technologies are currently being investigated to stimulate an economic and sustainable way of handling our food supply [6]. One of those by-products is created during the soymilk production and will be subject of this study.

OKARA, A BY-PRODUCT OF THE SOYMILK PRODUCTION

An increased awareness about health benefits associated with the consumption of soy foods, milk-related allergies, and orientation towards a more sustainable food production have led to an increased amount of soy products. Soymilk is one of those products, whose production and consumption has increased in most countries, and which again goes along with the accumulation of the by-product okara. Current soymilk production statistics are not widely available. In 1983 soymilk production was estimated to be close to 1 million tons [7], but has drastically increased within the last decades. In 2006, soy beverage production has reached more than 1 million tons in West Europe, North America, and Japan only [8]. Traditional soymilk is made with a soybean to water ratio of 1:5 (**Fig. 1**), though sweetened and flavoured soy drinks are made with a bean to water ratio up to 1:20. However, using a bean to water

ratio of dairy-type soymilk (3.5% protein content) of 1:7 [7], and assuming that per ton of soybeans 1.2 tons of okara are produced during the soymilk production [9], one can estimate that from 1 million tons of soymilk, about 170.000 tons of okara are produced. Tofu and other soy-based products are not included in this estimation, but also based on a primary soymilk production. Therefore, several million tons of okara produced yearly is a probable estimation [10-12]. Okara consists of about 80% water, and most of the insoluble components from the soybean. Due to its high moisture content, okara also contains water-soluble components. The exact composition of okara depends mainly on the soybean variety used and the processing conditions employed during the soymilk production, which typically consists of three major steps: soaking of the soybeans, cooking and grinding to prepare a slurry, and separating the slurry into soymilk and okara by mechanical means (**Fig. 1**) [7, 12-14]. Especially its moisture content depends on the separation method of soymilk and okara. Typically, okara has a moisture content around 80%, and a dry weight composition as shown in **Fig. 2**.

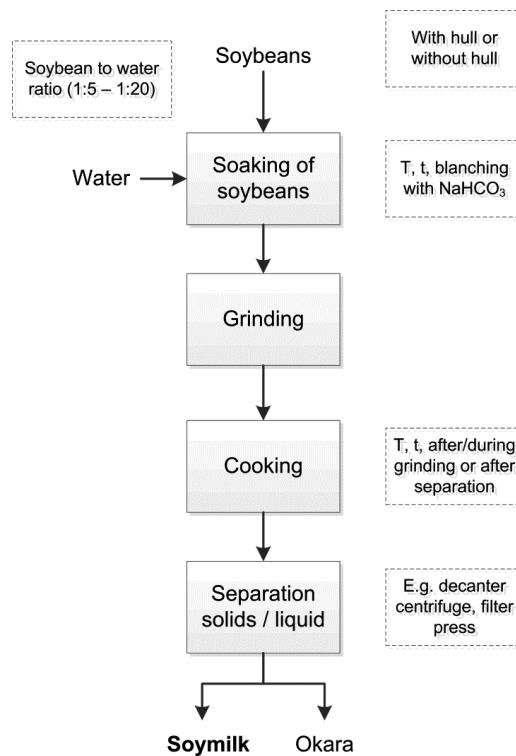


Fig. 1. Soymilk production (main processing steps and common variations)

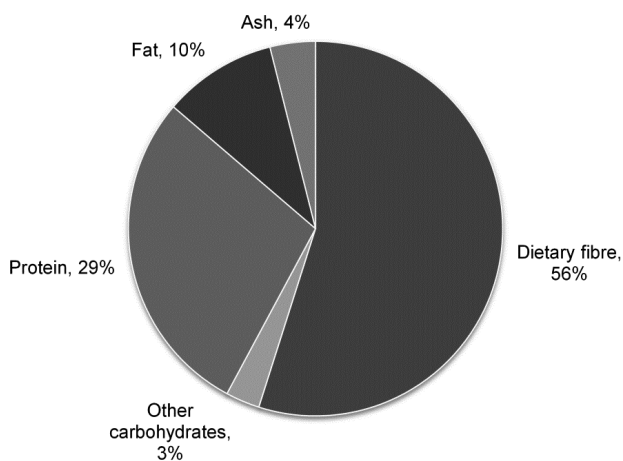


Fig. 2. Composition of okara (adapted from [15])

In literature, two main approaches to utilise okara for food applications are described. In the first approach, the entire okara is used as ingredient in food products. In the second approach, different constituents are isolated from the okara, and investigated for further use as ingredient.

Incorporation of okara as complete ingredient in food products often requires drying of the fresh okara. Okara is a material that is difficult to dry, because of its high tendency to agglomerate leading to poor spouting behaviour and a low energy efficiency of the drying process [16-18]. In a dry state, it is used to enhance the texture of a product, since it shows good water and oil retention capacity, or to enrich the product with nutritional components such as fibres and proteins. Bedani et al. [19] increased the nutritional and functional properties of soy yoghurt with the addition of okara, Lu et al. [20] partially replaced wheat flour by dry okara to make bread, steamed bread, and noodles having a lower glycemic index, and Waliszewski et al. [21] added okara to corn tortillas to improve their amino acid profile. Other examples of products containing the complete dry okara are a soy-based snack [22], a soy (nougat) candy [23], a Brazilian cheese bead [24], and a French bread [25].

Fewer authors attempted to add wet okara to a food product. Turhan et al. [26] and Su et al. [27] described its use to produce reduced fat beef burgers, or to lower the fat content in a coconut-based baked snack [28]. Furthermore, Rinaldi et al. [29] introduced wet okara in

cereal based products. Wet okara, due to its high moisture content, spoils very quickly. Hence, wet okara is difficult to use.

Regarding the utilisation of ingredients from okara, most attention has been given to the polysaccharide fraction of okara. The polysaccharides present in okara have been characterised in detail by Redondo-Cuenca et al. [15], Mateos-Aparicio et al. [30], and Li et al. [12] as the fibres in okara, which are mainly insoluble, possess high water and oil retention capacity, and high water swelling capacity. The indigestible fraction of the fibres show good suitability for fermentation by bifidobacteria, showing potential for use as prebiotic ingredient [31]. To increase the fibre solubility, okara has been treated with enzymes and high pressure processing (HPP) [32] or extrusion [10, 33]

Concerning the protein fraction of okara, earlier research focused on extraction and modification methods to make the protein in okara suitable as ingredient [34-36], whereas recent research focused on detailed characterisation and composition [37, 38]. Moreover, Vishwanathan et al. [39, 40] investigated the extractability of the protein in okara, and the production of a protein concentrate using membrane separation. A study about peptide production by protease hydrolysis showed an anti-oxidative activity of the produced peptides [41].

Finally yet importantly, okara was studied for its potential to deliver natural antioxidants. Methanol, acetone and water were used to extract valuable components such as polyphenols and oligosaccharides, which have antioxidant or prebiotic capacity [42-45]. Until now, isoflavones, being a specific type of polyphenol, did not get broad attention, even though they are present in considerable amounts [14], and represent a considerable potential value. Therefore, these isoflavones are the subject of this study.

ISOFLAVONES PRESENT IN OKARA

Isoflavones are present in many legumes, amongst which soybeans can be considered a major source, and their isoflavones are thus widely studied. The isoflavones' status as phytoestrogens made them a widely discussed topic in the literature, because they are suggested to play a role in e.g. hormone related cancers, osteoporosis, and postmenopausal syndromes [46-48]. Their specific effect on health remains to be investigated further though, since contradictory or inconclusive studies exist [49]. Nevertheless, since a large amount of

literature relates soy isoflavones with the mentioned health benefits, an increased effort to isolate the components and fortify food products has been made in research and development.

There are three basic isoflavones in soy (daidzin, genistin and glycitin), which can all three be in four different forms: the aglycones, the β -glucosides, the malonyl-glucosides, and the acetyl-glucosides. Isoflavones are polyphenols with a similar structure to flavones [50, 51]. The difference is in the position of one of the two benzene rings, which is attached to the C-3 carbon of the heterocyclic ring for isoflavones and to the C-2 carbon for flavones. Flavones and isoflavones are subclasses of flavonoids, one of the largest groups of polyphenols [50, 52, 53]. **Fig. 3** shows the four groups of isoflavones with their according three types of molecules. The total isoflavone concentration and the different isoflavone forms present in the soybeans and their products depend on the soy variety, its cultivation, the process and the storage conditions [14, 54-56]. The malonyl-glucosides and β -glucosides are the primary forms present in the soybeans, but those components can be transformed into aglycones and acetyl-glucosides during processing, due to enzymatic conversion or thermal stress [57]. The most common chemical changes in the isoflavone form include the decarboxylation of the malonyl- to acetyl-glucosides and the ester hydrolysis of the acetyl or malonyl-glucosides to β -glucosides. Furthermore, cleavage of the glucosidic bond leads to an increased amount of aglycones [56, 58].

From small-scale to large-scale production of soymilk and okara, manufacturers employ several methods, which can vary at several processing steps (**Fig. 1**). This change of processing conditions can affect the isoflavone profile in the resulting products. The most relevant differences are in the temperatures used during soaking of the soybeans, the hot- or cold-grinding of the soy slurry, the cooking temperature, and sterilisation before or after separating the soymilk from okara [9, 14, 29, 59-61].

Several researchers have studied the stability of those isoflavones. Chien et al. [62] studied the conversion and degradation (hydrolysis) of the four different genistin forms at 100, 150, and 200 °C, and showed that conversion and degradation occurred faster under moist conditions. Mathias et al. [63] investigated the conversion and loss of isoflavones at 25, 80, and 100 °C in combination with neutral, acidic, and basic conditions, and found that the malonyl- and acetyl-glucosides are more stable under acidic and neutral conditions than at pH 10, while the combination of 100 °C and pH 10 resulted in the highest conversion. Xu et al. [64] showed that the glucosides daidzin, glycitin, and genistin are relatively stable around the boiling point, but started to degrade above 135 °C, and drastically degraded at temperatures above 185 °C, with daidzin being more stable than glycitin and genistin. The aglycones

themselves were relatively stable up to 200 °C, again with daidzein having a higher stability than glycetein and genistein.

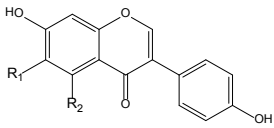
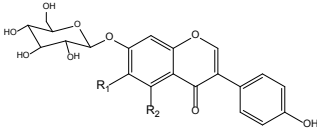
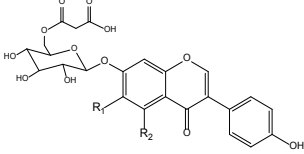
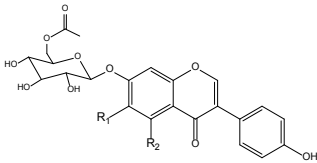
Group	Isoflavone form	R1	R2	Chemical structure
Aglycones	Daidzein	H	H	
	Glycitein	COH ₃	H	
	Genistein	H	OH	
β-glucosides	Daidzin	H	H	
	Glycitin	COH ₃	H	
	Genistin	H	OH	
Malonyl-glucosides	Malonyl-daidzin	H	H	
	Malonyl-glycitin	COH ₃	H	
	Malonyl-genistin	H	OH	
Acetyl-glucosides	Acetyl-daidzin	H	H	
	Acetyl-glycitin	COH ₃	H	
	Acetyl-genistin	H	OH	

Fig. 3. Structure of soy isoflavones

All studies described above used pure components. A food matrix generally has unpredictable influence on the behaviour of a single component in a multi-component system, since different components can interact with each other. Furthermore, their analysis is complicated by the complexity and the other components being present, and several parameters during processing procedures can have synergistic effects. Nevertheless, the influence of processing on isoflavone profiles in several types of food was investigated and reviewed by Villares et al. [65]. The isoflavone profile in okara mainly depends on the temperature and time employed during soaking and grinding of the soybeans, because the β-glucosidase activity around 45 °C

leads to an increased amount of aglycones, while prolonged heating around 100 °C or higher induces thermal hydrolysis of the most unstable form, the malonyl-glucosides [9, 56, 59, 66, 67].

Another factor that complicates the behaviour of isoflavones in okara is their interaction with other components in the matrix. Non-covalent interactions between polyphenols and macronutrients have been widely discussed in literature, and are thought to be due to weak associations based on hydrophobic interactions, probably enhanced by hydrogen bonding. The focus was on proteins and polyphenols for some time, but now also carbohydrates and lipids have been increasingly discussed for their potential interaction with polyphenols based on similar mechanisms [68, 69]. The effects of processing conditions such as pH and temperature on the partitioning of the isoflavones towards proteins have been investigated with the interest in isoflavone enriched soy protein isolates. Most of these studies indicate non-covalent interactions of hydrophobic nature or hydrogen bonding between the isoflavones and proteins [55, 70-74]. However, direct measurements of the binding of isoflavones and proteins is difficult, e.g. due to their low solubility [75].

POLYPHENOL ISOLATION

Polyphenols have been associated with beneficial properties for human health. These properties include e.g. preventative effects against cardiovascular disease and cancer [48]. Along with the increasing amount of literature about their beneficial health properties, the literature about their recovery and enrichment also increased [53, 76]. The exploitation of by-products for polyphenol recovery in this respect has gained special interest in the view of contributing to a more sustainable agricultural and food production [5]. In fact, by-products are often very rich in phenolic compounds, due to their location in peels and seeds, which are often retained in the residues. Their relatively low water solubility and their tendency to associate with other components may contribute to polyphenol-rich by-products. In addition to the reduction and utilisation of side streams, better processes (e.g. more energy-efficient, no toxic solvents) will contribute to more sustainable processing. Application of polyphenols can be manifold: as food flavours and colorants, as bioactive health ingredients, and as product stabilising antioxidants.

Numerous conventional and non-conventional technologies have been proposed for the separation of those high value components. Conventional solid-liquid extraction commonly uses hydroalcoholic mixtures [77], and an increasing popularity of ethanol can be noticed in

literature due to its food-grade and less toxic nature. However, many other solvents such as acetone, acetonitrile, methanol, and ethyl acetate as examples are still widely investigated for polyphenol extraction [77, 78] due to the relatively easy solubilisation that these solvents or solvent mixtures offer. Acid, alkali, and (sub or super) critical fluid extraction are common alternatives. Modern technologies, such as pulsed electrical fields, ultrasound-assisted, or microwave-assisted extraction have been suggested to increase the yields or to overcome some of the challenges in polyphenol extraction, such as kinetic limitations in extracting them from the cellular matrix, instability of the components, or solvent residues in the final product. For the purification stage, the major principles used generally are membrane separation and adsorption. The principles of adsorption have found application as affinity chromatography to purify the components out of a liquid, but this is a time, labour, and solvent consuming technology.

The procedures to isolate isoflavones generally involve large quantities of solvents (e.g. ethanol, methanol, isopropanol, butanol, hexane, or diethylether) and often, multiple steps are required in the process. For instance, the requirement of multiple chromatography columns makes the production on industrial scale complex. Other steps include crystallisation, membrane filtration, or high-cost hydrolysing enzymes. Furthermore, many of the methods include heating, strong acids, or organic solvents, which cannot only have a detrimental effect on the desired and remaining components, but certainly have a negative impact on the environment [79-82]. In most cases, the laboratory scale processes are too cumbersome for efficient industrial application. A popular starting material for isoflavone production is soy molasses, the by-product of soy protein concentrate production, because it contains isoflavones in a slightly more concentrated form due to being the aqueous alcohol extract of soybean flakes. However, many patented processes are using soybeans or soy flour as starting material, and the recovery of isoflavones from side streams such as okara would require less valuable resources.

Only few separation processes from by-products have yet found application in the industry, because the cost efficiency remains yet a challenge [4-6, 83]. The fact that those micronutrients are usually present in low amounts in a complex matrix (< 1%) makes it particularly challenging to separate them in a cost efficient and environmentally-benign manner. Furthermore, the interaction of polyphenols with other components in the matrix seems to complicate the separation process, and the enormous structural variation in polyphenols, despite having a similar backbone, makes it difficult to find a suitable solvent/separation process, which fulfils the requirements on cost, environment and efficiency.

PROCESS SYNTHESIS

Sustainability in processing in the food industry is an emerging topic, but requires structured methodologies to support the development of more economic and sustainable processes. Process synthesis methodologies and conceptual process design have been widely used in the chemical and pharmaceutical industry to meet a combination of objectives such as product quality, safety, and costs. Another benefit of systematic design approaches is the reduction of the product-to-market time frame. A rapidly changing market and continuously changing objectives, such as new regulations, economic situations, but most importantly increased sustainability concerns, ask for simultaneous co-development of those methodologies [84, 85].

Process synthesis methodologies generally follow either of two approaches, the hierarchical approach, and the mathematical approach [84, 86, 87].

In short, the mathematical approach involves a formal, mathematical representation of the problem with subsequent optimisation of the constraints and objectives, which have been defined *a priori*. This optimisation-based method uses algorithmic methods to find the optimal solution to the design problem, and has found wide application for example in heat exchanger networks or chemical reaction paths [88-90]. A major drawback of this method is the tremendous computational effort that is needed, especially with increasing number of degrees of freedom. A process can be designed choosing from a number of operations, their sequence and connectivity, but also the detailed operational setting of each operation. Therefore, finding the optimal solution is only possible by severely limiting the parameter space beforehand. Especially in systems where a complete capture of all the details of product properties and quality during processing is difficult, such as with foods, this approach is not realistic [88, 91, 92].

The hierarchical approach uses long-term experience and so called heuristics, and is especially useful if there is a lack of data to describe the problem by models, or the structure and properties are difficult to predict by thermodynamic models due to heterogeneous systems such as food [82]. The method aims for ‘close-to-optimal’ solutions by going through several levels, where local decisions are made at each step. The general requirements are defined in an input-output structure, after which this structure is successively defined in more and more detail. With this, the designer goes from a very rough network of physical or chemical transformations all the way towards a complete, feasible flow sheet alternative [87, 88].

The analogy between complex chemical consumer goods and structured food products [91, 93, 94] led to the development of product-driven process synthesis (PDPS) [95], a methodology based on the hierarchical decomposition approach of Douglas [87]. The PDPS methodology was established for the rapid development of processes for structured food products such as mayonnaise, ice-cream, or margarine. It divides the complexity of the process design into several hierarchically ordered levels, and rapidly produces feasible process alternatives.

This approach for the production of structured food products has shown successful application leading to feasible, simplified processes with reduced costs for emulsions [96], to improved consumer attributes in the design of bouillon cubes [97], and the significant reduction of water and energy usage [98]. The PDPS methodology has however been developed and to date only applied to combine ingredients for the production of a structured, composite product, and has not yet been used for the isolation of components from a complex, structured matrix. As this isolation is roughly the reverse of the creation of a structured matrix from ingredients, very different decisions have to be taken: the material structure and composition will determine the presence of thermodynamic (solubility or partitioning) or kinetic (diffusion) limitations; stabilisation by pasteurisation or sterilisation is less important, auxiliary media such as extractants may be required, and residues of these materials in the depleted material may be a consideration.

Therefore, we here have set out to consider the methodical synthesis of ingredient isolation processes, especially where these ingredients need to be isolated from a concentrated, complex matrix. There are many examples of these, but an important category that has economic relevance is the extraction of polyphenols from by-products. To make use of such systematic procedure possible, we here investigated the applicability of process synthesis to the case of the separation of isoflavones from okara.

AIM OF THIS THESIS

Modern food processes have to simultaneously comply with ever stricter environmental restrictions, have to be more efficient and complete in their use of raw materials, have to severely limit the use of energy, water and auxiliary materials (e.g., solvents), and at the same time should deliver high-quality, safe products, for a minimum of costs.

While the effectiveness in creating high-quality products and in reducing costs has obtained ample attention, the systematic incorporation of the sustainability of the process in the synthesis approach (i.e. the efficiency in using energy and water, the emissions to the environment, and the minimisation of waste) has obtained less attention.

The use of low value by-product streams for the isolation of valuable components is a first step towards a more sustainable food value chain, and thus deserves a place in the field of process synthesis. A systematic approach is required to develop more efficient and mild technologies with non-toxic solvent routes. Okara is a highly nutritious by-product from the soymilk production and has a semisolid complex matrix. The isolation of isoflavones from okara is in this respect a good case to study.

The aim of this thesis was therefore to investigate principles and mechanisms that underlie the separation of isoflavones from okara, and to evaluate the applicability of a process synthesis methodology to support the development of a sustainable and economic separation process for isoflavones from a complex matrix, based on these principles and mechanisms. As discussed, this was done using the isolation of isoflavones from okara as an example *in casu*.

THESIS OUTLINE

Chapter 2 reports on the behaviour of the components and the matrix in the extraction of isoflavones with different solvents. The focus was on ethanol and water, and mixtures of those, as relatively environmentally friendly solvents. The diverse affinities of different isoflavone groups towards ethanol-water mixtures are described, as well as the swelling of the matrix due to its high fiber content. The possibility to use the water that is captured by the fibres as extraction solvent is discussed.

To assess the sustainability of the extraction process, an exergy analysis was carried out in **chapter 3**. Streams of different nature are compared by thermodynamic means, which gave further direction within the process synthesis. The analysis revealed that a large improvement

on the system could be made by using water in the pre-treatment of the matrix. Furthermore, the glycosides of the isoflavones were found to be rather water-soluble. Therefore, the behaviour and extraction mechanisms of all isoflavone groups in the water environment were investigated in **chapter 4**. In this chapter, we describe the affinities of the isoflavone groups towards the matrix and the water with a model, and discuss the possible effect of protein-isoflavone interactions, as well as the effect of ionisation of the components during separation.

Chapter 5 discusses the use of affinity as principle to separate the isoflavones from a water extract. The water-insoluble polymer PVPP was used to adsorb the isoflavones, and their adsorption at different pH was described with a model. The influence of the matrix on such adsorption process was investigated, and a mechanism for simultaneous extraction and adsorption is discussed.

The conceptual design of a process can be supported by process synthesis methodologies, and the use of a process synthesis methodology is presented in **chapter 6**. The insight gained during this research was used to conceptually design a sustainable process for isoflavone separation from okara, supported by product-driven process synthesis. A relatively simple water-based extraction-adsorption process with subsequent elution with ethanol for purification is suggested.

Chapter 7 summarises the findings, and sketches the areas in which we still need to develop the methodology further. It concludes with a discussion about other process alternatives, and some guidelines for future developments.

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Chapter 2

The potential of crude okara for isoflavone production

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Abstract

This study describes the extraction of isoflavones from crude okara, a by-product from soymilk production, using industrially relevant conditions. Ethanol and water were chosen as environmentally friendly and non-toxic solvents. A wide range of ethanol concentrations was tested (0-90% ethanol) for extraction at room temperature. It was shown that the extraction of isoflavones was possible from crude okara. This creates opportunities for a more attractive extraction process regarding industrial processing, as the energy-consuming step of drying could be omitted. The optimal concentration of isoflavones in the extract was reached with ethanol concentrations between 50% and 70%. Ethanol concentrations above 60% required an elevated liquid-to-solid ratio due to the high moisture content in okara. Increased ethanol concentrations lowered the protein content, which corresponded to an increase in purity. A high water content in the solvent resulted in co-extraction of a larger amount of other components.

INTRODUCTION

The production of soymilk is associated with the production of its by-product okara. Okara is the insoluble part after hot water extraction of the soybeans and contains predominantly fibres, protein and fat [1-3]. Several attempts have been made to add okara into food products such as candy or soy snack foods to fortify those products with nutritious components or to improve textural properties [4, 5]. Other authors explored its use as substrate for fermentation to produce valuable components such as citric acid or the lipopeptide antibiotic iturin A [6, 7]. Okara can also be used directly as a food product, such as Okara tempeh, which is commonly used in Indonesia, although the production of okara greatly exceeds the amount currently used for food consumption, and okara's acceptance as a food product is limited to few parts of Asia. Therefore, most okara ends up as animal feed or even waste [7-9].

An alternative approach to upgrade okara is by separation into more valuable components. In recent years, the recovery of bioactive components from agricultural by-products has become an important research area [10]. Despite being lower than in other soy based foods, the isoflavone concentration in okara is considerable [11, 12], which makes it attractive to study whether okara can be upgraded by extraction of those valuable components. Typical starting materials used for industrial isoflavone production are soy molasses, soy germ, or soy flour, where isoflavones are already present in concentrated form [13]. Isoflavones are components belonging to a group of polyphenols having a high potential economic value, due to the fact that research on isoflavones suggests that these components are largely responsible for the suspected health benefits of soy [14, 15]. Therefore, isolation and purification of isoflavones are of high interest and may increase the economic potential of okara.

Soybeans contain up to twelve different types of isoflavones, classified in three main groups with each four forms, namely aglycones, glucosides, malonyl-glucosides, and acetyl-glucosides. Okara can contain the same twelve isoflavones, although the processing conditions during the soymilk and okara production will change the original isoflavone profile of the soybeans [16]. For example, Wang and Murphy [17] showed that more aglycones are present in the okara than in soymilk. Furthermore, malonyl-glucosides decrease with a concurrent increase of glucosides and aglycones during heat application and soaking steps [12, 16, 17]. Typically the isoflavone content in okara ranges between 12% and 40% of the original content of isoflavones in soybeans. This leads to an isoflavone concentration that ranges from 0.02% to 0.12% in okara (d.w.), depending on the type of soybeans [9, 12, 17].

Many authors studied the extraction of isoflavones from soy foods using different solvents such as aqueous methanol, ethanol, and acetonitrile, acidified or non-acidified, or with dimethyl sulfoxide [18-20]. Just as different solvents are used in analytical methods to quantify isoflavones, several solvents (e.g. methanol and ethyl acetate) and isolation methods for isoflavones can be found in patented preparatory processes [21-23]. However, for industrial application, ethanol and water remain the preferred solvents because of environmental and health reasons.

Utilising a by-product and using mild solvents are important steps towards more sustainable processing. Compared to the other soy-based products, okara is characterised by its high water content of about 80%, which makes it susceptible for fast microbial and chemical spoilage. It is therefore common to dry it prior to further processing. Additionally, dry material usage allows a free choice of solvents and solvent ratios during the extraction. Drying this large amount of water in okara on the other hand, will be energy consuming and might cause thermal damage to the okara [24-28]. In case the extraction is performed with a certain amount of water (e.g. with the use of aqueous ethanol), it could be beneficial to make use of the water in crude (wet) okara and omit a drying step. We therefore use the crude, wet okara as a source of isoflavones. Water-ethanol mixtures are used as solvent, because aqueous ethanol has also shown satisfactory results for isoflavone separation [29, 30], and is well accepted by consumers. However, the use of water-ethanol mixtures entails a dilemma, especially when trying to keep the amount of solvent used as low as possible, which is important when industrialising the extraction. As stated before, okara has a water content of about 80%. That water will become part of the extraction solvent during processing. In case a solvent with an ethanol concentration of 80% is required, the amount of ethanol to be added should be 4 times the amount of water present in okara. This leads to a liquid to dry solid ratio of 20. This is also illustrated in **Fig. 1**, where it is shown that it is not possible to achieve a liquid-to-solid ratio of 10 with ethanol concentrations higher than 60% (wet extraction) without first removing some of the water.

The objective of this paper therefore is to investigate the primary extraction of isoflavones from crude okara using different ethanol-water concentrations. The potential of using crude instead of dried okara will be evaluated, and the consequences for future process design will be discussed.

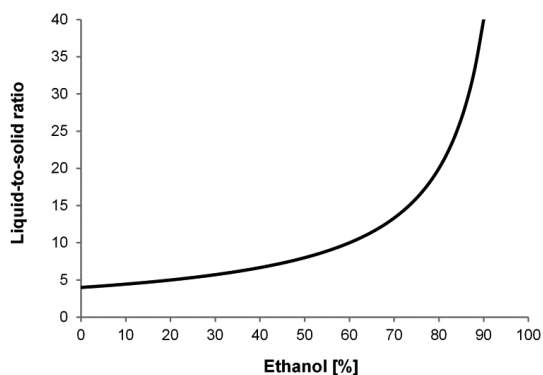


Fig. 1. The minimum liquid-to-solid ratios necessary for extraction using crude okara with a moisture content of 80% at various concentrations of ethanol (0-90%). 0% ethanol is equivalent to pure water.

MATERIALS AND METHODS

Materials

Okara was produced as described in the following section. The dry matter content of the okara was 22%. The isoflavone concentrate Novasoy[®] 700 (ADM, USA) was provided by Unilever. Isoflavone standards: daidzin, glycitin, genistin, daidzein, glycitein, genistein, acetyl-daidzin, acetyl-glycitin, acetyl-genistin, malonyl-daidzin, malonyl-glycitin, and malonyl-genistin were purchased from Nacalai USA Inc. (San Diego, USA). The standards were dissolved in analytical grade DMSO (Sigma Aldrich Co., Schnelldorf, Germany). Solutions containing all 12 isoflavones standards were prepared using methanol (Sigma Aldrich, USA) and stored at -20 °C. All solvents were HPLC grade.

Milli-Q water (Q-Gard 2 Purification Pack, Millipore, France), ethanol (Sigma Aldrich, USA), methanol (Sigma Aldrich, USA), acetonitrile (Biosolve BV, The Netherlands), and formic acid (Sigma Aldrich, USA) were used as solvents and eluents for HPLC analysis.

Okara production

The okara was produced with an ASC50 soymilk system (ProSoya Inc., Ottawa, Canada). Soybeans were soaked for 2h to soften the beans before adding the mixture into a cooker grinder vessel in a ratio 7:1 (water:soybeans). After grinding, the slurry was heated up by

steam injection and cooked for 3min at 105 °C. Then, the slurry passed through a deodoriser vessel to remove undesired volatiles responsible for the beany flavour of the soymilk. At the outlet the soy slurry had a temperature around 80 °C. A filter centrifuge separated the fibrous by-product okara from the soymilk. Around 1.5kg okara was produced per kilogram of soybeans. Okara from the same production batch was used for all experiments. One part was freeze-dried, the rest (crude okara) was stored at -20 °C in sealed containers until analysed. Photos of the extracts (**Fig. 2**) are representative, but taken during the same experiment with okara produced with a pilot plant at Unilever (Unilever, Vlaardingen, The Netherlands).

Extraction

Crude okara

Approximately 4.5 g of crude okara was used for the extraction. Ethanol and water were added to the samples in extraction tubes. The extraction tubes were shaken to mix each sample thoroughly, and afterwards placed in a rotator (IKA RW20) (240/min) for 2 h at room temperature to complete the extraction of isoflavones.

Water and ethanol were added to the sample to obtain aqueous ethanol solutions of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 wt% ethanol, respectively. 0% ethanol is equivalent to pure water. A dry liquid-to-solid ratio of 10 to 1 was used for most samples, although, 70%, 80%, and 90% aqueous ethanol were obtained by increasing the liquid-to-solid ratio to 12, 17, and 35 to 1, respectively.

The same procedure was followed for comparison of solvents using water with acetonitrile, methanol, and ethanol, respectively. For those extractions, the solvents were mixed in such ratio, that a 50% solvent concentration and a dry liquid-to-solid ratio of 20 to 1 was obtained.

After extraction, the samples were centrifuged for 30 minutes at 24,010g and 4 °C (Beckman J2-MC centrifuge). The extract and the pellet were separated and kept at -18 °C until further analysis. The weights were recorded accurately during all steps.

Freeze-dried okara

Okara was dried with a Christ GmbH Epsilon 2-6D freeze-dryer. 1 g of dry sample was mixed in an extraction tube with 10g of solvent (0-100% ethanol), and subsequently treated the same way as the crude okara.

Novasoy solubility test

20 mg of the commercially available isoflavone concentrate Novasoy[®] 700 was mixed with 10 g of 0-100% ethanol, respectively, to reach an isoflavone concentration in the same range as in extracts from okara. Tubes with the mixture were stirred for 30 seconds with a vortex (IKA MS1 minishaker), and afterwards placed in a rotator (IKA RW20) (240/min) for 2 hours at room temperature. For the HPLC injection, 50µl of the mixture was diluted in 1g of the same solvent, respectively, and left in a shaker (Eppendorf[®] Thermomixer) for 30min to equilibrate.

HPLC analysis

Isoflavone content was analysed by Reversed-Phase High Performance Liquid Chromatography (HPLC). Aliquots of the extracts were filtered through a 0.2 µm filter before HPLC analysis. The HPLC system was equipped with an autosampler (Finnigan Surveyor Plus, Thermo Scientific), a pump (Thermo Scientific), and a photodiode array detector (UltiMate 3000, Dionex). A Waters Atlantis dc18 column (3 µm, 2.1 x 150 mm) was used to separate the isoflavones with a column temperature of 40 °C. The autosampler was kept at 10 °C during measurements. A gradient was composed of the following two eluents: 0.1% formic acid in Milli-Q water and 0.1% formic acid in acetonitrile. The solvent flow rate was 0.3 ml/min. Standard solutions containing all 12 isoflavones in pure form were used for calibration and identification. Concentrations of the calibration curve were 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 10, 25, 50 µg/g, respectively. The peak areas of the standard solutions and the extracts were measured using Dionex Chromeleon 7 Chromatography Data System, Thermo Scientific (Massachusetts, USA), at a wavelength of 254 nm.

Dry matter content

The dry matter content of the okara was analysed using AOAC Method 925.10 (AOAC 1998). The extracts were dried overnight in an oven at 40 °C and 40 mbar, and the weight losses were recorded.

Protein content

The nitrogen content of the dried extract, freeze-dried pellet, and freeze-dried okara was determined by Dumas (Thermo Scientific FlashEA 1112 Analyser). Methionine was used as a standard, and a conversion factor of 6.25 to calculate the amount of protein in a sample.

Isoflavone concentration and purity

The isoflavone concentration in the extract was calculated by multiplying the concentration measured with HPLC with the dilution ratio and correcting for the density of the different solvents. The purity was then calculated based on the isoflavone concentration in the solvent, which was multiplied by the amount of extract obtained and divided by the dry matter content of the extract. The results have been given as percentage.

Statistical analysis

All experiments were carried out in triplicate. Data was analysed by analysis of variance (ANOVA) using IBM SPSS version 20 (SPSS Inc., Chicago, USA). Differences within the group were determined using Least Significant Difference (LSD) multiple comparison analysis. Differences at a p-value < 0.05 were considered significant.

RESULTS AND DISCUSSION

Comparison of different solvents

The recovery of isoflavones from okara with different solvents is shown in **Table 1**. The highest value for the isoflavone concentration was reached with 50% acetonitrile, though the difference with 50% ethanol was not statistically relevant ($p > 0.05$). The concentration of isoflavones was lowest in 50% methanol. The optimal solvent depends mainly on the polarity of the extractant and extracted components, but can furthermore depend on other factors, such as the matrix, the viscosity of the solvent, etc. In the case of the result expressed in total isoflavones, the optimal solvent will also depend on the isoflavone profile present in the matrix [31]. Listed in elution order of the HPLC analysis, the following isoflavones were present in the okara extracts: daidzin, glycitin, genistin, malonyl-daidzin, malonyl-glycitin,

malonyl-genistin, daidzein, glycitein, acetyl-genistin, and genistein. Acetyl-daidzin and acetyl-glycitin were not found, which is in line with previous research [9, 12, 17]. Therefore, the total isoflavone content will refer to the 10 isoflavones present. It can be concluded that isoflavones in okara dissolved easily in ethanol, which makes it the solvent of choice for industrial applications given the fact that it is food-grade and relatively environmentally friendly.

Table 1. Recovery of isoflavones from okara using different solvents

Solvent	Total isoflavones (µg/g)
50% Acetonitrile	923.4 ± 14.1 ^a
50% Methanol	849.9 ± 25.42 ^b
50% Ethanol	907.4 ± 7.8 ^a

Results are expressed as means ± standard deviations (n=3).

Results with the same letter are not significantly different ($p > 0.05$).

Extract impurities and residue swelling

The use of a mixture of ethanol and water does not only solubilise isoflavones; the okara matrix swells, and some other components may actually be solubilised or dispersed as well. **Fig. 2** shows that with water and 10% ethanol, the extract is milky white, indicating that not only other components are dissolved, but part of the matrix is dispersed in the extract. Apparently, centrifugation at 24,010g for 30 min was not sufficient to remove the suspended material. With higher ethanol concentration, the extract became much clearer, meaning that it contained less undissolved solids. **Fig. 3** shows the matrix swelling. The residue after extraction with water and 10% ethanol remained highly swollen, which can be attributed to okara's high fibre content [32]. With water or 10% ethanol, the okara absorbs 90% of the liquid in case of a liquid to solid ratio of 10 to 1 (**Fig. 3**). With 20% ethanol, the pellet contains around 4 parts liquid to 1 part dry matter, which is almost equivalent to the moisture content of natural okara. Therefore, extraction with water and 10% ethanol did not release more extract than water present in okara before extraction. The solid content of the 60% ethanol extract increased from 1% up to 3% in the water extract. One can thus see that a high-water solvent induces strong swelling of the matrix, but also liberates a significant amount of solids into the extract. It is evident that this has impact on the design of a potential extraction process. The liquid-to-solid ratio has to be kept to a minimum for industrial applications, regarding costs and recovery processes for the solvent. The swelling of this high fibrous product has to be considered. High swelling of okara due to high water content in the solvent and extremely low liquid-to-solid ratios might impede processes such as mixing and transportation.

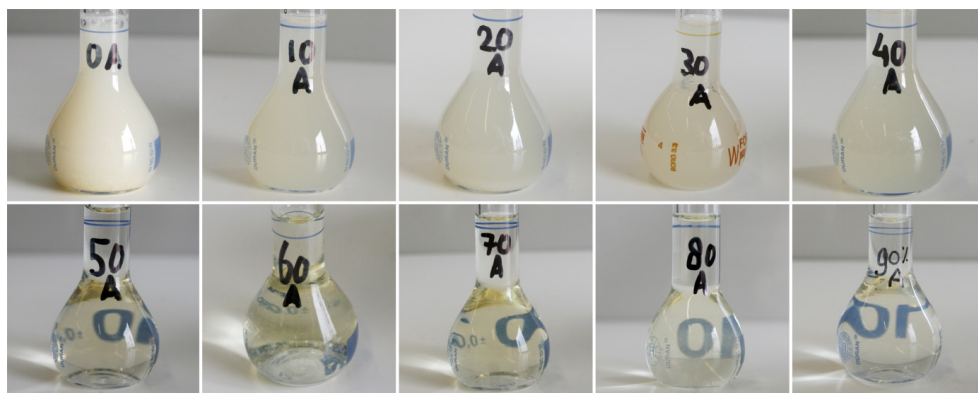


Fig. 2. Images of isoflavone extracts from crude okara, obtained by employing various concentrations of ethanol (0-90%). 0% ethanol is equivalent to pure water.

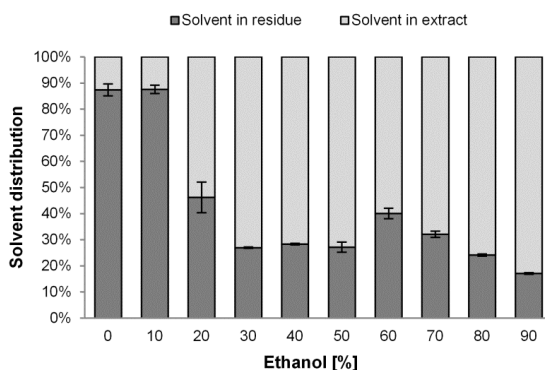


Fig. 3. Solvent distribution depending on the ethanol concentration after centrifugation at 24,010g and 30 min. 0% ethanol is equivalent to pure water. Error bars indicate standard deviations.

Extractability of isoflavones from crude and dry okara

Fig. 4 shows the influence of the solvent quality on the extractability of isoflavones from okara. In **Fig. 4a**, the total isoflavone content extracted from dry and crude okara is shown, considering all 10 isoflavones present in the okara, whereas **Fig. 4b** shows the results for the separate groups: the aglycones, glucosides, and malonyl-glucosides, respectively. Acetyl-genistin accounted only for maximal 3% of the total isoflavone concentration, and is therefore

not shown in **Fig. 4b**. For extraction from crude okara, about the same extraction results were obtained with ethanol concentrations between 50% and 80% (around 900 µg total isoflavones/g), whereas at low and at high ethanol concentrations the isoflavone content in the extract decreased. Water, 10%, 20%, 30%, and 40% ethanol yielded 372, 463, 565, 759, and 834 µg/g isoflavones, respectively, and 90% ethanol yielded 836 µg/g. The different ethanol concentrations performed differently for each group of isoflavones (**Fig. 4b**). The extract with pure water hardly contained any aglycones; the aglycone concentration increases slowly with increasing ethanol concentration and reaches a plateau value at 60% ethanol and higher. With pure water, the most extractable forms were the malonyl-glucosides, followed by the glucosides. The malonyl-glucosides have their peak at slightly lower ethanol concentrations (30-60%) compared to the glucosides (50-70%). The decrease in total isoflavone concentration starting at 80% ethanol is predominantly due to the malonyl-glucosides and the glucosides.

To compare wet okara with dried material, part of the crude okara was freeze-dried and subsequently extracted. The results after extraction with 0-100% ethanol from the dry material are also shown in **Fig. 4a**. The trends of the two curves are similar, except at higher ethanol concentrations. Furthermore, the highest isoflavone concentration of the dry extraction is reached rather with ethanol concentrations from 40-60% instead of 50-80% as in the wet extraction. For both dry and wet extraction, the use of 50 and 60% ethanol resulted in high extraction yields, meaning that the use of crude okara does not have a negative effect on the extractability of isoflavones. The extraction from dry and crude okara with 50% ethanol was similar to the extraction from crude okara using more solvent (**Table 1**). No significant difference ($p > 0.05$) between a ratio of 10 to 1 and 20 to 1 could be observed, which indicates that the majority of isoflavones was extracted with the concentration of 50% ethanol at a liquid-to-solvent ratio of 10 to 1. Nevertheless, from 70% ethanol and higher, a difference between dry and wet extraction is shown (**Fig. 4a**). One should bear in mind that from 70% ethanol onwards, the liquid-to-solid ratio had to be increased for the extraction from crude okara. Higher isoflavone concentrations in 70, 80, and 90% ethanol extracts from crude okara compared to dry okara extracts may result from better solubilisation of the components at higher ratios. However, the isoflavone concentration drastically decreased until 100% ethanol for the dry extraction, which indicates that the matrix is the limiting factor at high ethanol concentrations. The water, which is already present in the crude okara, very likely keeps the matrix accessible for the solvent, whereas the water content at 90 and 100% ethanol during dry extraction is limited.

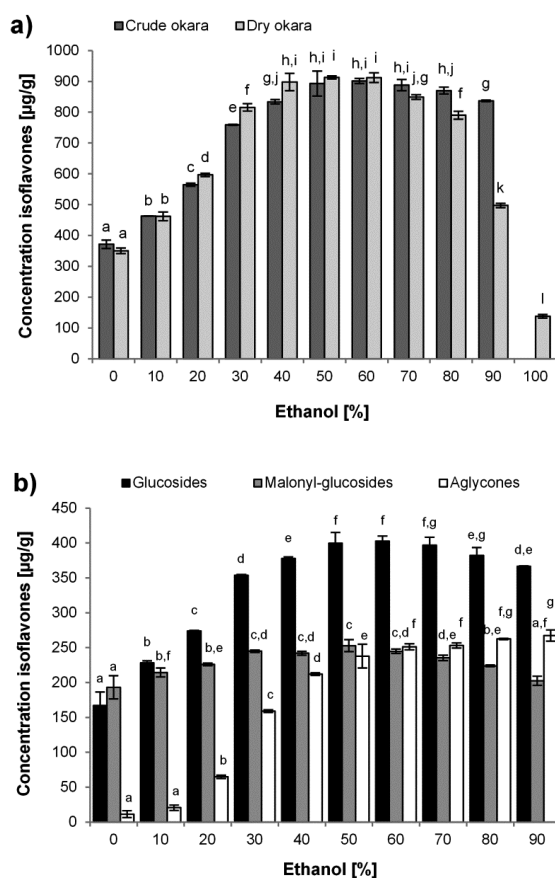


Fig. 4. The influence of different ethanol concentrations on the extractability of isoflavones ($\mu\text{g/g}$ solvent). (a) total isoflavone content extracted from crude okara and freeze-dried okara (2h extraction, room temperature), (b) aglycones, glucosides, and malonyl-glucosides extracted from crude okara. 0% ethanol is equivalent to pure water. Error bars indicate standard deviations. Bars with the same letter within one series (total isoflavones, glucosides, malonyl-glucosides, or aglycones), not across isoflavone groups, are not significantly different ($p > 0.05$).

Isoflavones are considered water-soluble components. However, they are less soluble in water than in slightly less polar solvents [29, 33]. The glucosides and their malonyl and acetyl derivatives with many hydroxyl groups are naturally more water-soluble than their corresponding aglycones, which explains the aglycones' low extractability at low ethanol concentrations (**Fig. 4b**). Matching the polarity of solute and solvent seems to be crucial, but the solvent also needs to open the structure of the matrix to allow diffusion of the components from the matrix to the liquid phase. Though not soluble, the fibrous matrix is polar, leading to

high swelling with water, but less swelling with ethanol. It might even be the case that high ethanol concentrations extract the remaining water from the matrix, which could then be highly viscous or possibly even glassy, inhibiting out-diffusion of the isoflavones. This effect might contribute to the fact that higher ethanol concentrations do not lead to further increase of the extraction yield. Intermediate concentrations of ethanol (40 – 70%) then give a good balance between solubility and matrix opening by swelling. Therefore, we suggest using the water present in the material as part of the extraction solvent, which is possible for intermediate concentrations up to 60%, according to **Fig. 1**. Lower ethanol concentrations might lead to a solubility limit of the isoflavones. Elevated temperatures can improve the solubility of isoflavones in water [34], while higher liquid-to-solid ratios may solubilise more isoflavones in water as well.

To investigate the solubility of isoflavones in different solvents, a commercially available soy isoflavone mixture Novasoy® 700, mainly consisting of glucosides, was dissolved in 0-100% ethanol in water (**Fig. 5**). The only clear solutions were obtained with concentrations between 40% and 90% ethanol; other mixtures lead to turbid suspensions with subsequent formation of a precipitate. One can see that the solubility is significantly lower at 0% (i.e. water) and 10% ethanol, where only 60% and 88% of the highest concentration was reached, respectively. We can therefore confirm that the lower extraction at low ethanol concentration is also due to the lower solubility of the glycosylated isoflavones. However, in the okara extract the effect of low solubility of total isoflavones was enlarged by the non-soluble aglycones.

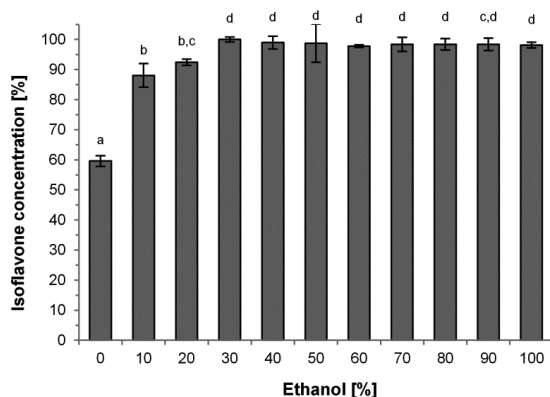


Fig. 5. Isoflavone concentration after dissolving 20mg Novasoy® 700 powder for 2h in 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% ethanol, respectively. 0% ethanol is equivalent to pure water. Error bars indicate standard deviations. Bars with the same letter are not significantly different ($p > 0.05$).

Fig. 5 not only shows that the solubility is limited when using low (<10%) ethanol concentrations, but it also shows higher solubility of the isoflavones in 90% and 100% ethanol. One should note that **Fig. 4a** shows a considerably lower yield of the extraction with 90% and 100% of ethanol. This further supports the hypothesis that here the extraction is limited by the matrix availability due to low swelling and more viscous solvent. Xu et al. (2006) found similar behaviour of isoflavones from red clover flowers in different ethanol concentrations, and Rostagno et al. [30] tested different ethanol concentrations for the purpose of optimising an analytical pressurised liquid extraction system, with similar trends regarding isoflavone concentrations at different ethanol concentrations.

Purity of the extracts

The purity of the extracts, determined by the selectivity, is an important parameter in any extraction process. **Fig. 6** shows the isoflavone concentration based on the total dry matter content of the extracts obtained and the isoflavone concentrations shown in **Fig. 4**. The purity of isoflavones increased with the ethanol concentration up to 80% ethanol. The isoflavone concentration in okara was around 900 µg/g (see **Fig. 4a**). Therefore, a concentration from 0.09% in the okara (d.w.) to 1.4% (d.w.) in the extract corresponds approximately to a fifteen fold concentration. 30% ethanol resulted in a concentration of 1% (eleven fold concentration), whereas 0% (water) to 10% ethanol resulted in a concentration factor of 2 and 4, respectively, compared to the concentration of isoflavones in okara. **Fig. 7** shows the protein content of the residue after extraction, and of the dried extract. The protein content of okara (d.w.) was $31.0 \pm 0.7\%$. The protein content of the extracts decreased with increasing ethanol concentration. This figure corresponds well with the pictures in **Fig. 2**. The decreasing protein content also corresponds well with the higher purity. The increased protein concentration of the pellet after extraction with 90% ethanol leads to the assumption that not only proteins, but other components from the matrix dissolved in this high ethanol concentration. The latter and the low isoflavone concentration in the 90% ethanol extract therefore lead to the low purity of the 90% ethanol extract as shown in **Fig. 6**.

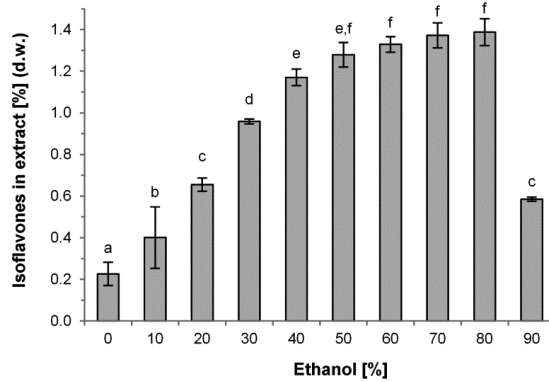


Fig. 6. Isoflavone concentration (%) in dry extracts. 0% ethanol is equivalent to pure water. Error bars indicate standard deviations. Bars with the same letter are not significantly different ($p > 0.05$).

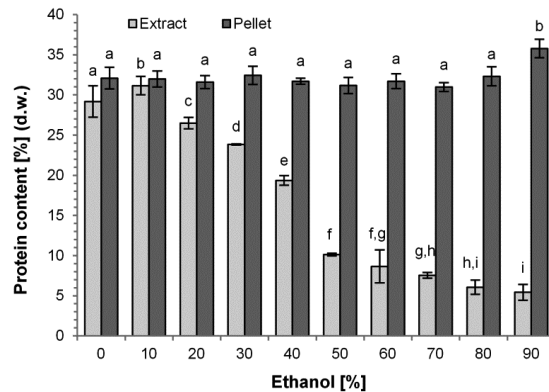


Fig. 7. Protein content in residues and extracts. 0% ethanol is equivalent to pure water. Error bars indicate standard deviations. Bars with the same letter are not significantly different ($p > 0.05$).

The potential of crude okara for isoflavone production

The consumption of solvents has a high impact on energy efficiency of separation processes and large amounts of solvent are detrimental to the efficiency of a process [35]. To make okara processing more sustainable regarding energy efficiency, we used the water that wet okara contains after being produced during soymilk production, as part of the extraction solvent for isoflavone extraction. Isoflavone extraction from soy products can be done with

aqueous ethanol. Water from the wet okara and relatively pure ethanol were combined to obtain the right water-ethanol ratio.

Solid-liquid extraction employs a solvent to dissolve and remove a soluble fraction from insoluble solids. In case of extraction from biomaterials, cells walls have to be disrupted to allow mass transfer of the solute through the material into the solvent. During the soymilk production process, the soybeans are already milled, and the production process simply comprises an extraction of the milled soybeans with water, and the majority of the isoflavones will be extracted into the soymilk [12, 17]. This explains the relatively low amount of isoflavones in the fibrous okara, which was around 0.09% in this study, which value is in line with previous research [11]. It is clear however, that the exact isoflavone content will depend on the starting amount of isoflavones in the soybean variety and the conditions during soymilk production [12]. The low starting amounts put high efficiency requirements for the isoflavone recovery process from okara. An advantage could be the fact that isoflavones are readily available in the crude okara. Okara contains 80% moisture in which a large part of the isoflavones might be present in addition to the isoflavones bound to the solid matrix. These facts suggest that the extraction of isoflavones from okara involves different mechanisms than a common extraction of components for example of soybeans. With a solvent in which the isoflavones are more selectively and better soluble, the isoflavones are easily separated from the okara. This explains why in additional experiments no effect of extraction time (5 min until 16 h) and temperature (5° C until 50° C) was found on the extraction yield, nor did a second extraction cycle extract more than what was left in the residue of the solvent if a good solvent (50-70% ethanol) was used (data not shown). This suggests that the majority of isoflavones left in the okara can be separated with a rather simple process, and utilisation of okara in this way has great potential.

Fig. 4 shows that the isoflavone concentration obtained with subsequent water extraction of the okara is relatively low, since other components are extracted as well (**Figs. 2 and 7**), while the more hydrophobic isoflavones (the aglycones) are not extracted at all (**Fig. 4b**). As **Fig. 6** shows, selectivity is increasing up to 80% ethanol. In the ethanol extracts, fewer components solubilise along with the isoflavones. According to Khare et al. (2006) the major impurities in alcoholic extracts from soy are saponins, oligosaccharides, and proteins [36], as for the latter also shown in **Fig. 7**.

Okara is a processed material. This most likely makes components, which are originally locally separated from each other in the living system, get in contact, and possibly interact [37]. Polyphenol-protein interactions are often described in literature, where hydrogen bonds

and hydrophobic interactions between the polyphenol and macromolecule are for instance responsible for the non-covalent weak associations between those two molecules. Solvents such as ethanol can inhibit the interaction between polyphenols such as isoflavones and proteins [37]. Therefore, it is hypothesised that the presumed water-soluble isoflavone forms are less available in the water phase than in the ethanol phase due to their interactions with the okara (proteinaceous) matrix, leading to lower extraction yields.

The okara had a considerable amount of aglycones. Often, more aglycones are present in the okara than in soymilk, probably due to their lower solubility in water compared to their corresponding glucosides [16, 17]. Furthermore, soaking time and temperature during soymilk production can influence the level of aglycones in a soy system [38]. Finally, initial cold-grinding may as well have led to an increased amount of aglycones due to prolonged β -glucosidase activity [39]. Therefore, the extractability in different solvents largely depends on the way okara was processed and the amount of aglycones present. Intensive processing leads to less water-soluble forms, and thus a lower extractability with water.

The liquid-to-solid ratio was set to 10 in this study. Lower ratios were practically not possible when water was used as solvent due to the high swelling capacity of the okara [32]: ratios of 5 or 6 allowed no separation of the solid and extraction liquid at higher water concentrations, as the matrix then takes up all the solvent. In contrast, separation of the extract and the residue is easy with ethanol, even at low liquid-to-solid ratios. However, using both a lower ratio combined with a high concentration of ethanol for the highest purity and little protein, implies that okara would have to be dried (see **Fig. 1**). When crude okara is used, the focus should be on lower ethanol concentrations to decrease the solid-to-liquid ratio. Solvent recycling, which is expected to be incorporated in such process, could compensate partly for higher solvent use in case higher ethanol concentrations are required, for example to create higher purities. Nevertheless, larger streams, and the probable loss of some ethanol during distillation, may lead to a less efficient process. Reducing the moisture content of the crude okara with methods mentioned by Choicharoen et al. [26, 27], Li et al. [40] or Wachiraphansakul and Devahastin [28] may be another option. The moisture content would only need to be reduced until the desired ethanol concentration can be reached with a lower liquid-to-solid ratio. Here, a more detailed analysis could clarify which process scenario leads to less environmental impact, a pre-processing step such as drying, or high solvent usage. Using pure water does not seem to be a good alternative at this stage, as it gives poor selectivity, and does not extract the aglycones. Even regarding the glucoside forms, the extractability was low, most likely due to interaction of the matrix with the isoflavones in water. The observation that addition of ethanol strongly improves the extraction supports the hypothesis that those interactions are

suppressed when ethanol is used. Nevertheless, the isoflavones chemical form and literature [41, 42] indicate that there is potential for improvement of the solubility of isoflavones in water.

CONCLUSION

To conclude, the study suggests a more efficient process for isoflavone extraction if the crude okara is used instead of dry material. No drying even keeps the isoflavones readily available in the water phase present in crude okara. The use of pure water however, does not lead to full extraction, most likely due to the fact that the aglycones do not solubilise, and that the binding between the glycosylated isoflavones and the matrix is not affected. Ethanol facilitates the extraction and reduces protein being extracted leading to higher yields and purity, though, this effect diminishes above 50% ethanol and the yield decreases. Therefore, 50% ethanol seems a good solvent as it leads to good extraction yields and does not require additional solvent use or drying of okara.

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Chapter 3

Exergy driven process synthesis for isoflavone recovery from okara

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Abstract

Isoflavones, found in soybeans and other members of the *fabaceae* family, are considered bioactive components of high economic value. An opportunity would be to separate isoflavones from okara, the by-product of the soymilk and tofu production. Such a process would not only valorise that side-stream but also, and maybe more importantly, reduce the waste of high quality bioactive compounds. Extraction is an important part during the recovery of isoflavones from okara and was conceptually designed in this work. Due to environmental constraints, ethanol and water were the only solvents considered in this work for extraction of isoflavones. Different process scenarios were established and assessed by solvent footprinting, energy use, and exergy analysis. Simulation of the various process scenarios showed that distillation and the loss of ethanol in the spent okara represent the largest inefficiencies regarding exergy waste and energy usage. Furthermore, even though the use of ethanol leads to a higher recovery, water is in most cases the preferred solvent due to the high exergetic cost of losing some ethanol in the spent okara and during distillation.

INTRODUCTION

The interest to utilise by-products in the food industry has drastically increased in recent years. The use of by-products not only increases the economic value of raw material: an increasing world population calls for alternative food sources and a zero-waste strategy. Lately, okara and its components have been thoroughly studied, and have shown high potential to be used in food products [1-5].

An increased awareness about health benefits associated with the consumption of soy foods, milk-related allergies, and orientation towards a more sustainable food production have led to an increased amount of soy products. Especially soymilk consumption has increased in most countries, which goes along with the accumulation of the by-product okara. In 2006, soy beverage production has reached more than 1 million tons in West Europe, North America, and Japan only [6]. Dairy-type soymilk (3.5% protein content) is made by mixing soybeans and water in a ratio of 1:7 [7], and assuming that per ton of soybeans 1.5 tons of okara are produced during the soymilk production [8], one can estimate that from 1 million tons of soymilk, about 200.000 tons of okara are produced. Other countries are not included in this estimation, neither are tofu and other soy-based products, which are also based on a primary soymilk production. Therefore, in total, several million tons of okara produced yearly is a probable estimation [1, 9, 10].

Okara and its structure and composition are shown in **Fig. 1**. Okara comprises mainly the insoluble material from the soybean, thus, many celluloses and hemicelluloses. Those fibres have a high water holding capacity, leading to a semi-solid product with 80% moisture content. Okara is obtained through a centrifugation process (e.g. with a decanter centrifuge). Therefore, it will be difficult to remove more water by centrifugation or other mechanical means, leaving drying the only option for further moisture reduction. In addition to valuable components such as soluble and insoluble dietary fibres, protein, and fat, okara contains isoflavones [11, 12]. Isoflavones belong to a large group of polyphenols, which reportedly play an important role in human health [13]. Those so-called bioactive components are typically present in low amounts in foods, but have a high value due to their health promoting effects. The isoflavones' status as phytoestrogens made them a widely discussed topic in the literature, because they are suggested to play a role in e.g. hormone related cancers, osteoporosis, and postmenopausal syndromes [13-15], and an increased effort to isolate the components and fortify food products has been made in research and development.

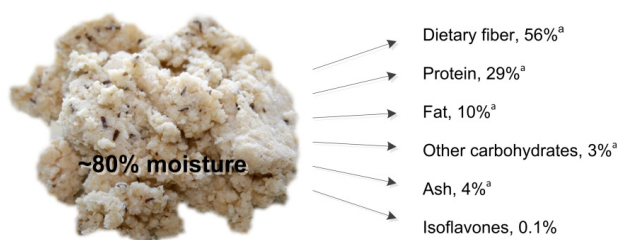


Fig. 1. Structure and composition of okara. ^a values according to [11].

Isoflavones represent about 0.1% of the dry matter of okara [12], and due to the large amount of okara discarded, a large amount of isoflavones is wasted every year. Being related to many health benefits, isoflavones have a high value for pharmaceutical and food applications in spite of their low concentrations in the food matrix. Therefore, their recovery from a by-product represents an interesting case. Given its status of a side-stream, the valorisation of okara requires the design of a specific separation process where the operational costs to extract a compound, present in low concentrations, must be kept to a strict minimum while preserving its functionality and quality. In addition to product quality, energy consumption, raw materials and investment costs, sustainability has become a factor with increasingly more weight during process synthesis [16].

In the process design considered in this work, okara enters the process with its original moisture content of about 80%, and is subsequently dried. Solvent is added to extract the isoflavones, and the isoflavone extract is separated from the spent okara. After separation of the solvent and the extract, the solvent is recycled from the isoflavone extract back to the extraction operation, thus concentrating the isoflavone extract. The concentrated extract is dried and an isoflavone product free of solvent is obtained.

The relatively simple process scheme described above already results in several alternative scenarios. Their configurations will vary in the extent of drying, and the type, concentration and amount of solvent used for extraction. The load of the most energy demanding unit operations (such as drying, distillation, and concentration) depends on the solvent quantity and quality, influencing the yield of the final product as well. Assessing the systems' sustainability is not trivial, since the optimisation of one unit operation might (negatively) affect the sustainability of the whole process. Further, sustainability evaluation is usually a subjective matter, where an evaluator can assign different priorities or importance to, for example, water footprint, energy consumption or the production of a waste stream. With the

present work, we show that a holistic multi-objective evaluation can be done with the aid of thermodynamic (exergy) analysis.

Cornelissen (1997) suggests that exergy analysis can be an important tool to analyse the sustainability of processes [17]. Exergy is an objective value calculated based on the first and second law of thermodynamics. Essentially, the exergy of a stream accounts for the potential work that such stream could perform when taken as a reservoir out of equilibrium with respect to a standardised environment [18]. It considers the quantity and the quality of the stream, depending on its potential energy, temperature, pressure, and composition. Nowadays, it is acknowledged as a holistic environmental indicator, since it has been demonstrated that minimisation of the exergy losses of a process is equivalent to minimisation of the resources needed and the emissions produced by such process [19, 20]. The concept of exergy is closely related to entropy. Although it is not possible to completely avoid the loss of exergy (increase of entropy) in real processes, it is nevertheless possible to minimise it. While physical exergy relates to the potential to perform work by heat transfer or expansion, chemical exergy relates to the potential of a stream to be used as a fuel or as a molecular building block. Therefore, while both are important in the overall process assessment, physical exergy loss is more related to inefficiencies in processing steps, while chemical exergy is more related to the efficient use of materials. Given that chemical exergies are generally orders of magnitude larger than physical exergies, and that they help identifying different kinds of inefficiencies, it is convenient to treat them separately.

Although exergy analysis has around 70 years of history, its application in food-related processes is relatively new [21, 22]. Drying, being an energy-intensive process unit in the food industry is an often-studied unit operation [23-26]. However, an exergetic analysis enables the process engineer to investigate as well entire production plants [27], or even entire agricultural systems including fertilisers and distribution of the products [28]. Widely used in the chemical industry, many recent applications can also be found in the area of ethanol production from biomass [29, 30]. The major focus has been on process optimisation achieved by exergy analysis, while fewer researchers have explored its use within process synthesis to determine *a priori* a process configuration that minimises the loss of thermodynamic potential [18, 31, 32]. The aim of this paper is therefore to approach the conceptual design of an isoflavone extraction process from the industrial by-product okara by using solvent footprinting, energy use, and exergy analysis. Furthermore, the use of exergy for sustainable process synthesis in the food industry is evaluated based on this extraction process.

METHODS

Calculations

An in-house model containing the mass, energy and exergy balance was built in Microsoft Excel® 2010. Scenarios were evaluated and compared based on their cooling water and steam consumption, energy consumption, exergy efficiency, exergy input, exergy loss, and exergy destruction.

An important feature of the model is the accurate consideration of the extraction yield of the isoflavones. In previous work [8, 12] we determined the impact of different solvent qualities (different ethanol/water ratios) on the concentration of the extracted isoflavones. At lower liquid-to-solid ratio, the polarity of the solvent dominates the extraction yield, with 50% to 70% ethanol leading to the highest yield of isoflavones in the solvent. The selectivity of the solvent for isoflavones (i.e., the obtained isoflavone purity) increases with ethanol concentrations up to 80% [12]. Pure water allows a simpler process without distillation of the solvent, but it leads to about half of the yield that is achieved with 70% ethanol. When water is used as solvent the yield can be improved with higher liquid-to-solid ratios [8].

Calculations around the distillation column are based on data of the liquid-vapour equilibrium of ethanol-water mixtures. More details on the distillation, specific features and the assumptions implicit in the model can be found in the Appendix.

The exergy balances were calculated according to the method developed by Szargut [33], considering physical (as function of temperature and pressure only), and chemical exergies (as function of composition). The chemical exergies were estimated from the tables developed by Morris and Szargut [34] when possible. An important exception was the chemical exergy of isoflavones, which was estimated to be 1.85 MJ/kg based on the heat of formation calculated with ChemBioDraw Ultra (v12.0.2.10.67, PerkinElmer). To facilitate the analysis, the lost exergy was assessed from the calculated exergy balance, and divided into destroyed exergy and wasted exergy. Destroyed exergy refers here to the loss of potential associated to a specific unit operation, and could be improved by a different choice of utilities, or by the choice of a different, better, and/or more innovative unit operation. Wasted exergy refers to the exergy lost in a wasted stream, such as cooling water or steam rejected to the environment, or a secondary stream produced as part of the process, which is wasted without further use.

Mass and Exergy flow visualisation

Sankey diagrams provide a clear overview of the mass and energy flows in a process [35]. Grassmann diagrams can be used for the representation of exergy flows in a system, and can be considered a sub-set of Sankey diagrams in which losses or streams dispersing into the environment are not represented. Dynamic Sankey and Grassmann diagrams live-linked to the model were created with e!Sankey Pro, (v2.5.2.2589, ifu Hamburg GmbH) for visualisation of the generated scenarios.

Process scenarios

Fig. 2 shows a schematic flow diagram to exemplify the base case scenario. The most important variables influencing the result of the analysis are the type of solvent and the liquid-to-solid ratio. The quality of the input solvent ranges from pure water to ethanol concentrations from 10% to 90% (discretised in increments of 10%). The liquid-to-solid ratios analysed range from 10:1 to 50:1 (discretised in steps of 10:1). The starting moisture content of okara was set to an average value of 81%. However, the dryer at the beginning of the process dries the material until a final moisture content, such that further solvent water needs are minimised. This feature becomes more important at low liquid-to-solid ratios and high ethanol concentrations. To reduce the degrees of freedom of the model around the distillation column, the bottom product was fixed to an ethanol concentration of 3%. The ethanol concentration of the top product was either set to 95% ethanol, or to a total minimum energy consumption around the distillation column. The upper recycle loop in **Fig. 2** represents the recovery of the ethanol. When pure water is chosen as solvent, the distillation unit is bypassed directly to the concentration step, and the lower recycle loop is used instead. The solid fraction of the spent okara was set to 0.5 based on typical experimental values. The importance of this value is discussed further below in section 0.

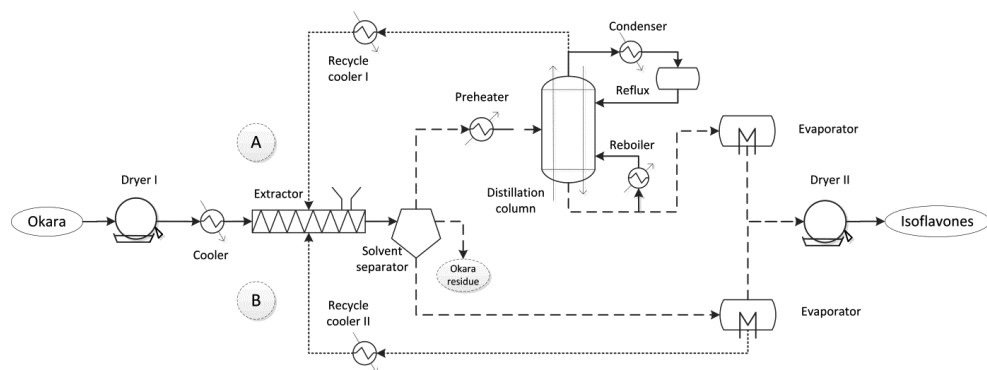


Fig. 2. Schematic flow diagram of the basic process. Dashed lines represent alternatives. Alternative A includes the configurations involving ethanol (10%-90% ethanol). Alternative B represents the configuration with water as solvent. Dotted lines represent the recycling of the solvent.

RESULTS

Visualisation with Grassmann diagrams

Graphical representations of exergy flows in the process with Grassmann diagrams (**Fig. 3** as an example) help the reader to pinpoint easily the locations of the highest losses of exergy. **Fig. 3** shows the Grassmann diagram for a 70% ethanol extraction at a liquid-to-solid ratio of 10. The calculations are based on 1 ton of crude okara (wet weight). In **Fig. 3**, the chemical exergy of the ethanol recycle loop (about 29.5 MJ/kg) was left out to help the viewer. Its magnitude is about ten times larger than that of the raw material, and therefore its inclusion would obscure the other streams and make interpretation unnecessarily difficult. The latter points to an immediate conclusion: the (exergetic) value of ethanol is so high that, if used, it should not be wasted, and its recovery is essential.

The analysis shows that the distillation process has one of the largest exergy losses. The other large loss occurs at the solvent separator. Fresh solvent has to be added to the process constantly due to losses at the distillation; in addition, the okara residue still contains solvent that has to be compensated for (set to 50% in the model). The solvent is not the only loss at the separator; also the okara residue has a high chemical exergy containing macromolecules such as carbohydrates, proteins, lipids and minerals, which are irreversibly lost if the okara residue is not further valorised.

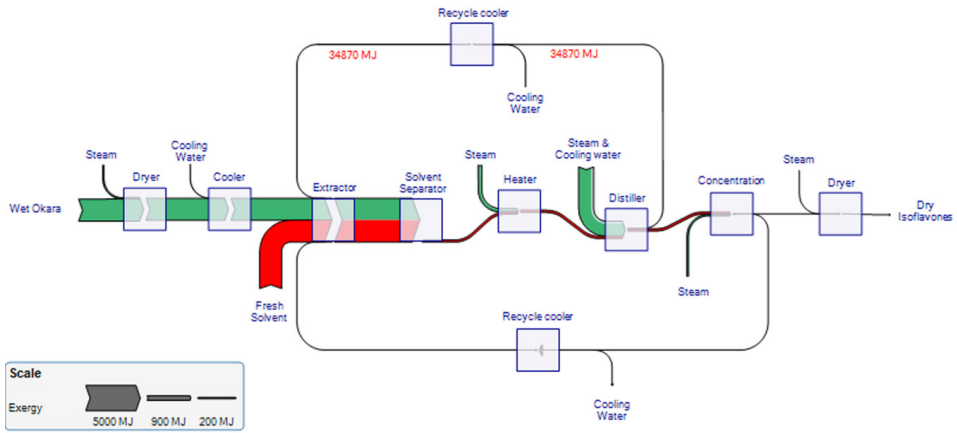


Fig. 3. Grassmann diagram of the extraction with 70% ethanol at a liquid-to-solid ratio of 10.

Fig. 4 shows the Grassmann diagram for a pure water extraction at a liquid-to-solid ratio of 10. The only large exergy losses occur at the solvent separator due to the extracted okara residue, and slightly smaller but noticeable losses at the concentrator and recycle cooler.

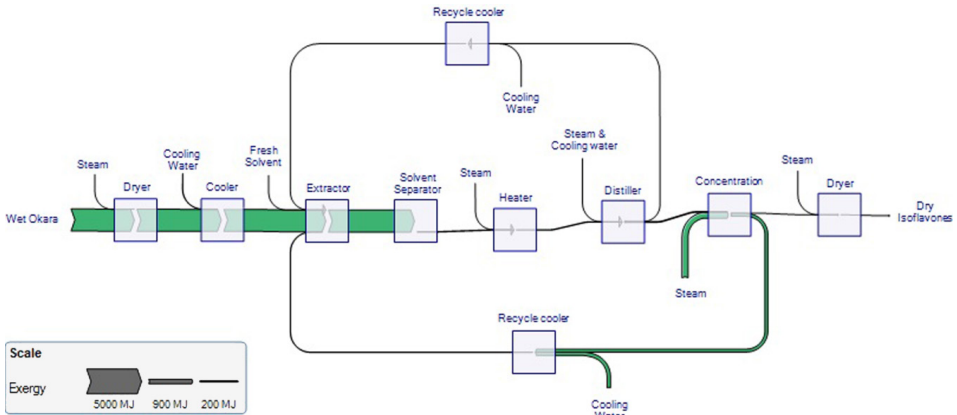


Fig. 4. Grassmann diagram of the extraction with pure water at a liquid-to-solid ratio of 10

Water usage, energy balances, and exergy analysis per kg isoflavones produced

This section discusses the valorisation of isoflavones as the sole product of the process. Therefore, the calculation basis for the sustainability indicators is 1 kg of isoflavones extracted. This implies that different amounts of okara are processed due to the different extraction yields. **Fig. 5 a)** and **Fig. 5 b)** describe the water usage and energy consumption as function of the ethanol concentration and solvent-to-solid ratio used for the extraction. The water usage has an optimum (minimum) around an ethanol concentration of 40%, while the energy curve shows the opposite trend, and energy consumption is highest for ethanol concentrations between 10-40% depending on the solvent-to-solid ratio. The energy consumption is lowest with water as a solvent or 90% ethanol.

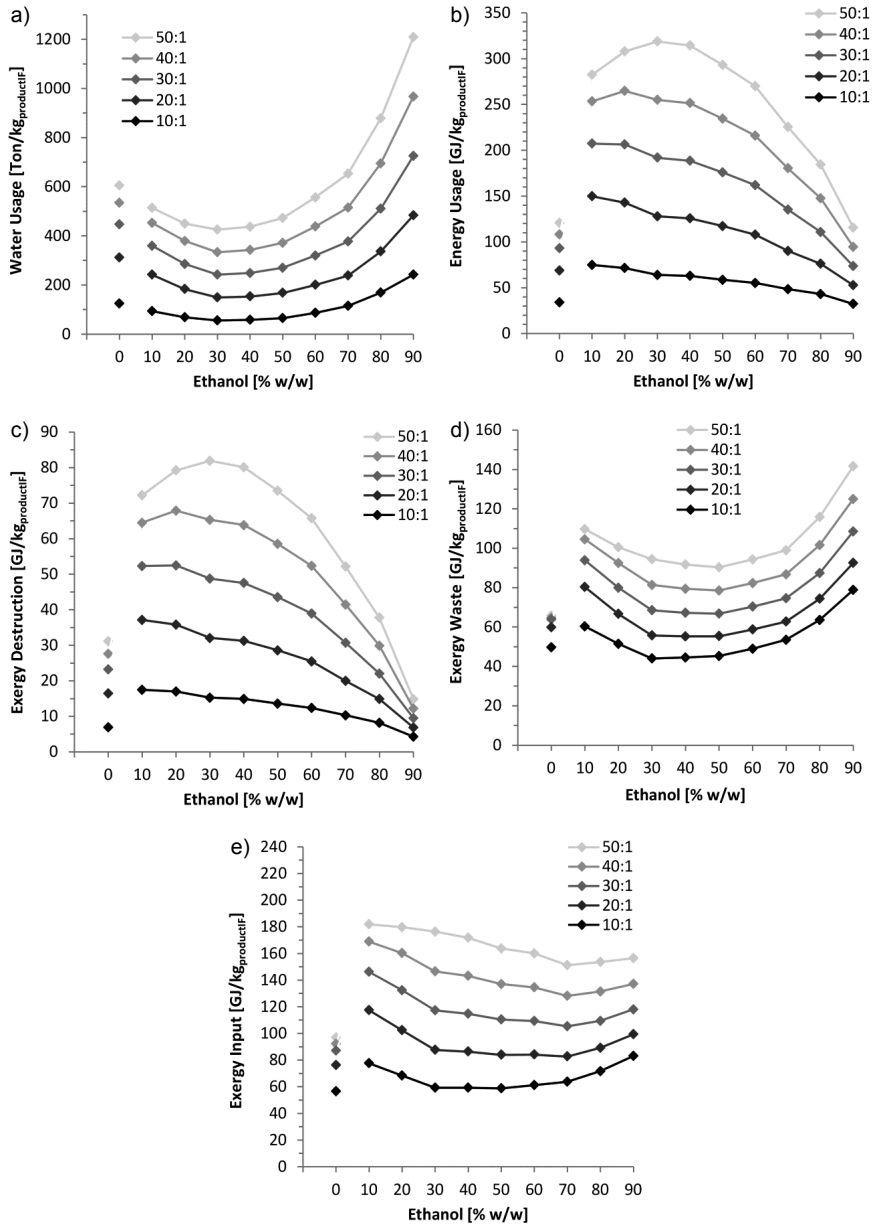


Fig. 5. a) Water usage, b) energy consumption, c) exergy destruction, d) exergy waste, e) exergy input, per kg of isoflavones produced, as function of the ethanol concentration and solvent-to-solid ratio. Head product of the distillation column is 95% in all ethanol extraction cases.

Fig. 5 c) - e) show the exergy destruction, the exergy that is wasted, and the total exergy input if the isoflavones are the only output of the process. The exergy destruction and the exergy waste together determine the total loss. In this case, the exergy destruction is smaller than the wasted exergy, typically around 50% of it. This difference gives a measure of the potential to improve the process by, for example, a different choice of hot and cold utilities, or by the replacement of a unit operation for a more sustainable one. **Fig. 5 c)** (exergy destruction) follows closely the same trend as **Fig. 5 b)** (energy consumption) with a bell-shaped curve with minima at 0% ethanol (water) and 90% ethanol. The exergy waste (**Fig. 5 d)**) is low, and stays low for the water configuration, also for higher solvent ratios due to the increased yield at higher ratios. However, if ethanol is introduced, the exergy waste increases, since the spent okara will retain some residue of ethanol. Furthermore, the distillation unit is introduced, but the yield at 10% ethanol is still very low. At higher ratios the loss of solvent (and high chemical exergy of ethanol) has such large impact that the increase of exergy waste at higher ratios is more detrimental. After a certain ethanol percentage is reached the yield of isoflavones does not improve, and the curves become parallel. At a liquid-to-solid ratio of 10, there is a minimum of exergy wasted for ethanol concentrations between 30% and 40%. At ethanol concentrations above 40% the exergy waste increases again, because exergy wasted at the solvent separator and distiller begins to dominate over exergy losses at the evaporator and recycle cooler II, which are higher at low ethanol concentrations than at high ethanol concentrations. From a solvent-to-solid ratio of 30:1, the water configuration produces the least exergy waste, because the exergy waste for the water configuration increases only at the evaporator and recycle cooler II for increasing ratios, but for the ethanol configurations an additional increase occurs at the distiller and heater.

Fig. 5 e) shows the exergy input for the different scenarios considering isoflavones as the only product, thus, the “embodied” exergy in a kg of product. It shows that the overall exergy input per kg product does not change much at a solvent-to-solid ratio of 10:1. Only a slight increase can be seen around 10%-20% and 80%-90% ethanol. As soon as ethanol is added in the process the exergy input increases due to the high chemical exergy of ethanol. This effect is increasingly noticeable at higher solvent-to-solid ratios. The minimum for the ethanol configurations moves towards higher ethanol concentrations as the solvent ratio increases due to the use of the first dryer, which increases the exergy input at high ethanol concentrations and low ratios. The sum of the exergy destruction (**Fig. 5 c)**) and the exergy waste (**Fig. 5 d)**) – the total exergy loss – is almost the same value as the exergy input, which indicates that the process in this configuration has an efficiency approaching zero. Overall, the results indicate that using water as solvent or using 90% ethanol gives lower exergy destruction values than

using intermediate ethanol concentrations. The exergy waste is lowest around 30%-50% ethanol, and in most cases the exergy input with water as solvent is lower or at least the same as for ethanol configurations.

In the previous case discussed (**Fig. 5**), the top product of the distillation was always 95% ethanol. To avoid drying of the okara, it is necessary to add relatively pure ethanol (95%) in order to reach certain ethanol concentrations without removing the water of the okara. However, distillation to high purity also requires a high energy input on the distillation column. Additionally, the exergy losses are high at distillation, due to the condensation of heating utilities and discharge of cooling water to the environment (shown as sudden changes of exergy in the Grassman diagram of **Fig. 3**). However, one can choose to obtain as head product of the distillation column an ethanol-water mixture of lower quality (i.e. lower ethanol concentration), with the consequence of the addition of higher amounts of fresh pure ethanol before the extractor. Therefore, there must be a trade-off between exergy efficiencies of the distillation column and the extractor. That is why new scenarios were generated considering minimum energy consumption in the distillation column to subsequently analyse the impact on the entire process. The outcome of the analysis is shown in **Fig. 6**. As more water is now available in the head product recirculated from the distillation column to the extractor, the target moisture contents at the dryer unit are lower, resulting in higher energy inputs for that pre-treatment. However, **Fig. 6** shows that the change of the distillation to lower ethanol production leads to an overall improvement, especially for the medium range ethanol concentrations. A general trend towards lower water and energy use and lower exergy losses/input is shown the higher the ethanol concentration. An exception results from 90% ethanol as solvent during extraction, where the ethanol has to be distilled to 95% and the column operates at the limit of its capacity. In this case, the minimum for the exergy input is not anymore found at water as solvent, but, depending on the ratio, at higher ethanol concentrations.

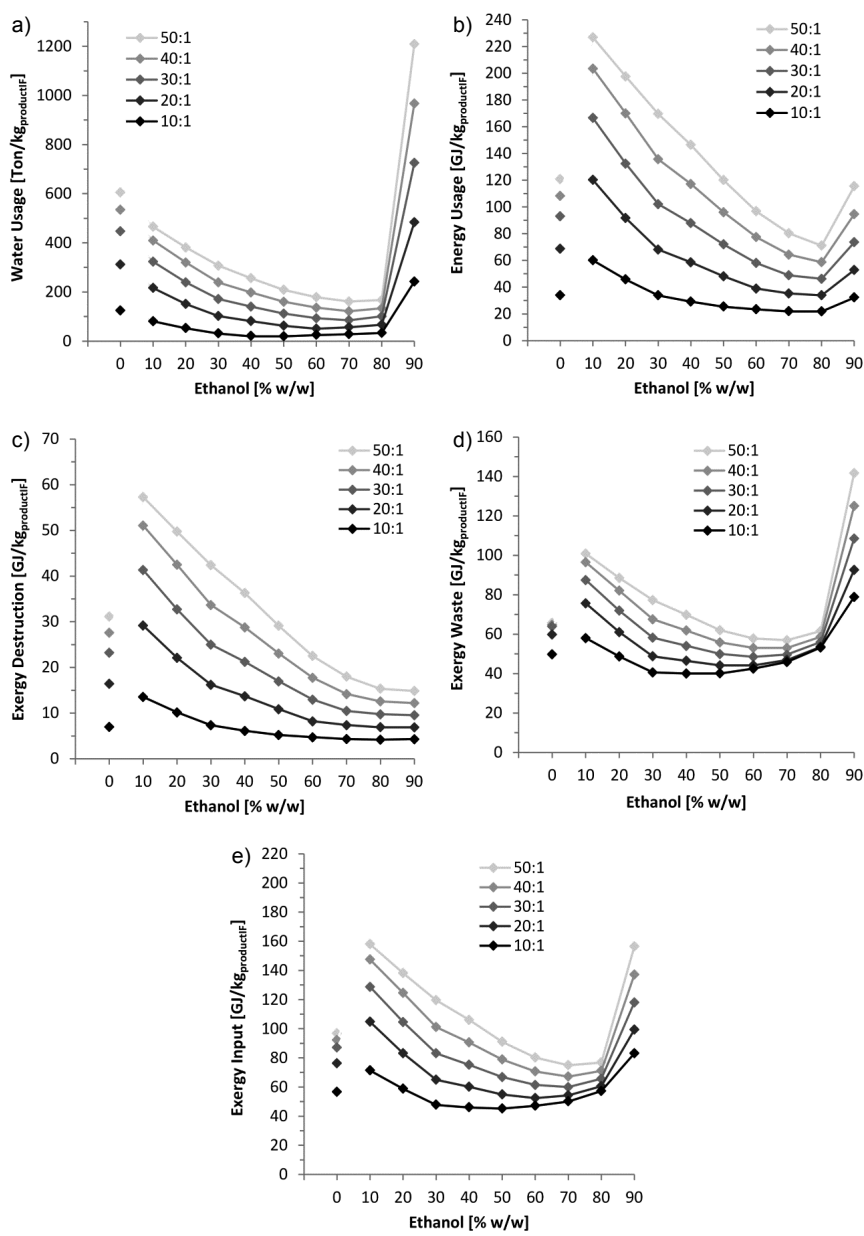


Fig. 6. a) Water usage, b) energy consumption, c) exergy destruction, d) exergy waste, e) exergy input, per kg of isoflavones produced, as function of the ethanol concentration and solvent-to-solid ratio. Distillation column is operated for minimum energy use in all ethanol extraction cases.

Water usage, energy balances, and exergy analysis per valorised solids produced

Fig. 3 and **Fig. 4** show that it is important that the spent okara after extraction of the valuable components is further used, e.g. as animal feed. When we assume this, it is most convenient to express the exergy used in the process per kg of valorised solids. The separation efficiency is nevertheless important, since the output (isoflavones + valorised solids) should have a higher value than the starting raw okara. Water usage, energy values, and exergy values are in this case reduced by approximately three orders of magnitude (**Fig. 7** and **Fig. 8** compared to **Fig. 5** and **Fig. 6**), but the trend stays in most cases the same. Considering that the waste at the solvent separator is valorised, the exergy efficiency of the process ranges between 33% and 69% (**Fig. 9**). The influence of the ethanol concentration on the efficiency is shown in **Fig. 9**. Also the overall efficiency of each conceptual process is improved when the distillation column is operated for minimum energy use (i.e. lower ethanol concentrations in the head product) despite the higher energy consumption of the first dryer. The efficiency decreases as ethanol concentration increases. However, the highest efficiency is found at 0% ethanol, i.e. using water as solvent. The difference between **Fig. 5** and **Fig. 7** indicates the large impact of the chemical exergy, mainly of the ethanol (around three orders of magnitude larger than the chemical exergy of water), but also the impact of the chemical exergy of the solids contained in the raw okara. Besides the general increase in efficiency if waste is valorised at the separator, the efficiency increases when less ethanol is lost. A major difference can be seen between **Fig. 5 d)/e)** and **Fig. 7 d)/e)**, the exergy waste and exergy input. Whereas the trends of the curves are similar for the other components of analysis, the exergy waste, and exergy input show different trends. The fact that isoflavones retained in the solids stream separated at the solvent separator are now also valorised makes the model insensitive to the separation yield of the components depending on the solvent quality. The immediate consequence of this is that water becomes the obvious choice as preferred solvent (**Fig. 7**). It furthermore shows that the exergy input depends on the ethanol concentration. However, one should bear in mind that the yield of the isoflavones with pure water is about 50% lower than with 50% ethanol.

The impact on the process of any ethanol loss can also be seen in **Fig. 8**, where a distillation operated for minimum energy use was considered. Also here, the exergy waste and exergy input increases the higher the ethanol concentration is in the solvent. However, this effect diminishes at higher liquid-to-solid ratios.

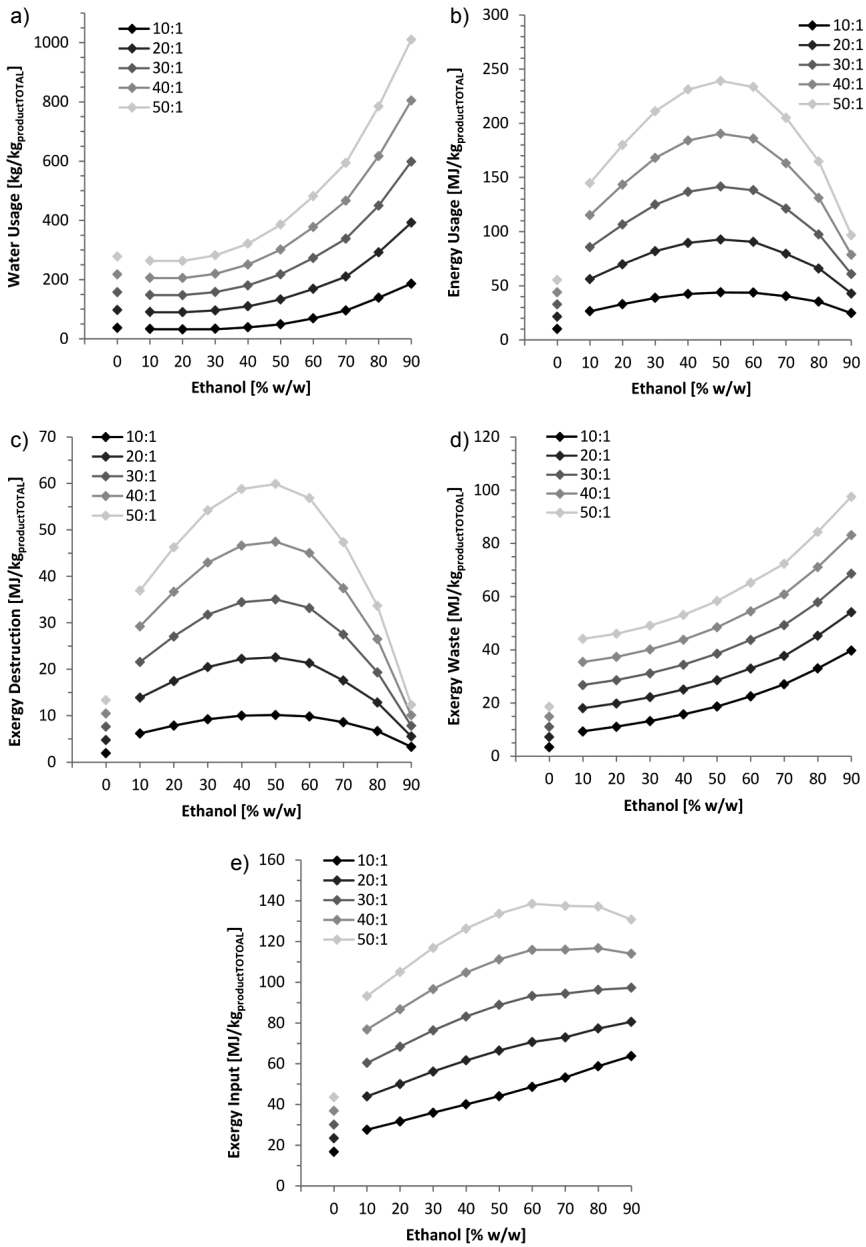


Fig. 7. a) Water usage, b) energy consumption, c) exergy destruction, d) exergy waste, e) exergy input, as function of the ethanol concentration and solvent-to-solid ratio, assuming the valorisation of the solids obtained from the solvent separator (ethanol in the residue is lost). Head product of the distillation column is 95% in all ethanol extraction cases.

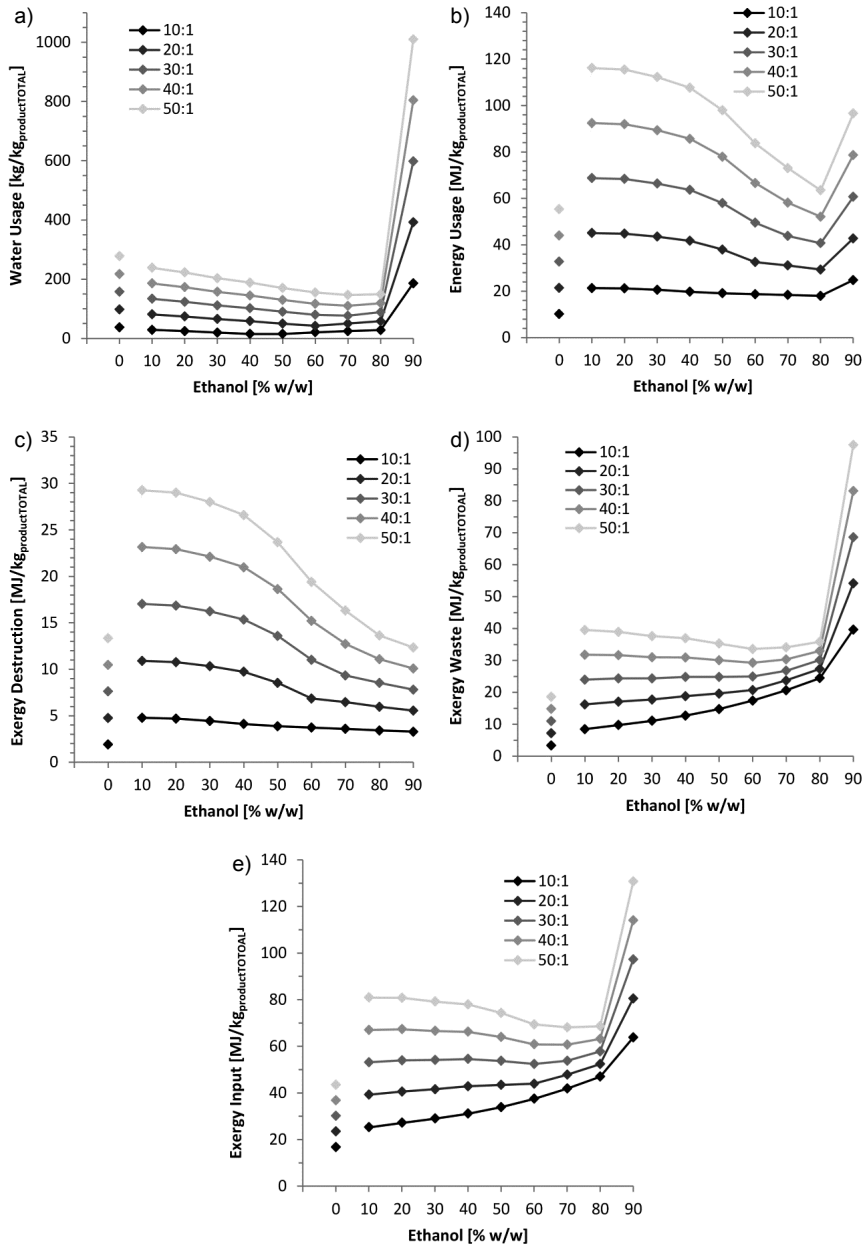


Fig. 8. a) Water usage, b) energy consumption, c) exergy destruction, d) exergy waste, e) exergy input, as function of the ethanol concentration and solvent-to-solid ratio, assuming the valorisation of the solids obtained from the solvent separator (ethanol in the residue is lost). Distillation column is operated for minimum energy use in all ethanol extraction cases.

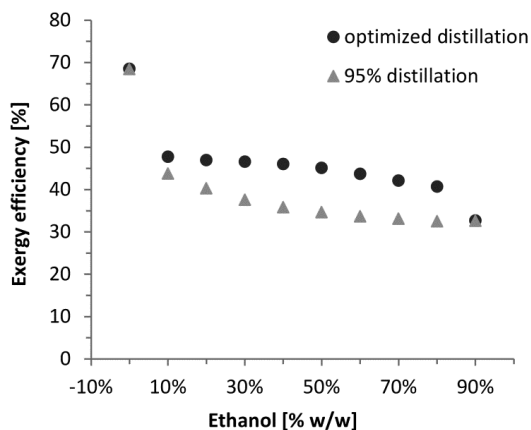


Fig. 9. Exergy efficiency in case the waste at the solvent separator is fully valorised. Liquid-to-solid ratio 10.

Effect of processing steps

Fig. 10 shows the exergy losses and their magnitude at the different processing steps for an ethanol concentration of 80% at different liquid-to-solid ratios. The motivation for this analysis is the trade-off between solvent quality, distillation column operation, fresh solvent need and the resulting target moistures required after the first dryer. The high moisture content of okara, and its fibrous, water binding structure makes its drying energy intensive. For water extraction, or ethanol/water extraction at low ethanol concentrations, one may avoid to dry this water and consider it part of the solvent [12], but to reach higher ethanol concentrations, without pre-drying the okara, one requires the addition of large amounts of fresh concentrated ethanol. Our model shows that, from an environmental point of view, it is better to pre-dry the okara and use lower liquid-to-solid ratios (**Fig. 10**) than to compensate with addition of extra fresh ethanol. Higher ratios and use of ethanol lead to increased energy expenditure at the distiller, and higher exergy losses at several stages. The energy and exergy losses at the first dryer instead have less impact.

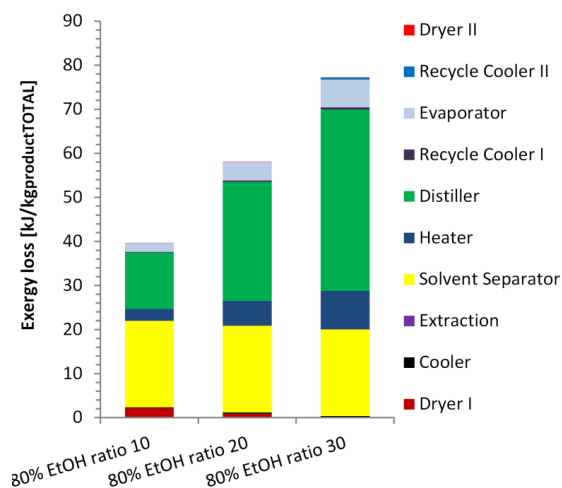
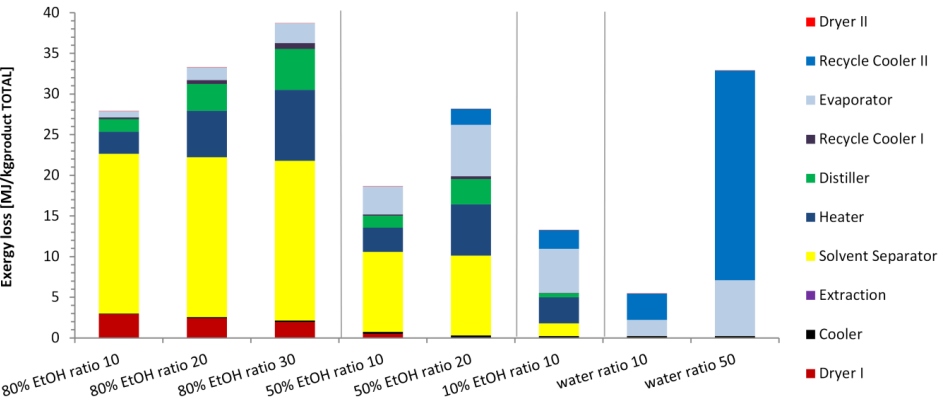


Fig. 10. Exergy loss of each step within the extraction process (per total solids valorised, head product of distillation set on 95% ethanol)

Fig. 11 shows the exergy losses and their magnitude at the different processing steps for ethanol concentrations of 80%, 50%, and 10%, at different liquid-to-solid ratios for the case of minimum energy use around the distillation. Furthermore, the exergy losses for two water configurations are shown, at liquid-to-solid ratios of 10 and 50. **Fig. 11 a)** shows the exergy losses calculated based on 1 kg of the total product (i.e. valorisation of the solids that leave the separator). It shows the high loss of resources at the solvent separator and high exergy losses at the distiller and heater. The more water is used in the process, the larger the exergy loss at the recycle cooler. The overall exergy loss is lowest for the water configuration at a low solvent ratio. The water extraction, even at high liquid-to-solid ratios, has lower exergy losses than some of the ethanol configurations at lower ratios. The exergy loss for the water configuration would decrease drastically if the water were recycled into the process, for example by using a membrane separation instead of evaporation and condensation of the steam with cooling water. **Fig. 11 b)** shows the results expressed per isoflavone yield (i.e. when isoflavones are the only product). In this case, the water extraction at higher solvent-to-solid ratios performs worse than the ethanol extraction at lower ratios. However, one can assume that exergy losses in this case can be improved by substituting the unit operation for the recycling of the water, as mentioned above. The water configuration at low ratio thus remains the one with lowest losses.

a)



b)

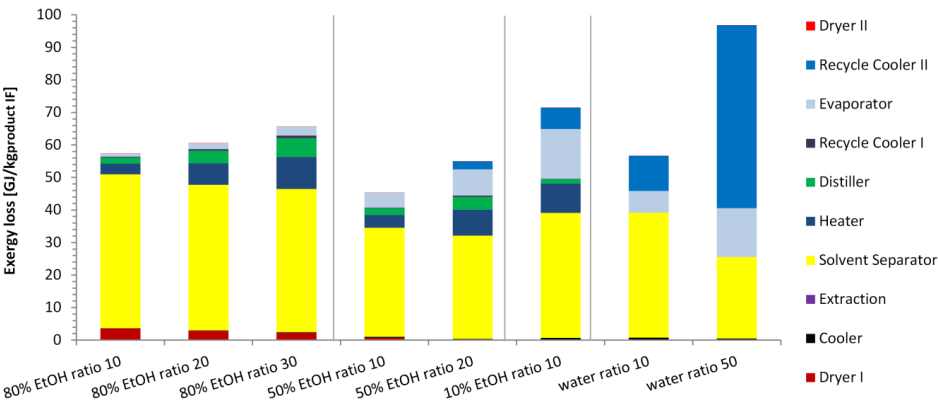


Fig. 2. Exergy loss of each step within the extraction process a) per total solids valorised, i.e. extract plus residue (in MJ), optimised distillation, b) per product (IF) (in GJ), optimised distillation

DISCUSSION

The outcome of the sustainability analysis is to a large extent driven by the chemical exergies of the raw materials. The large difference between the chemical exergies of ethanol and water defines most of the analysis. In addition, the chemical exergy of the dry fraction of okara has a relevant impact. The exergy losses are significantly reduced by choosing water as the preferred solvent for the extraction. If water is the only solvent, evaporation of water at the concentration step (see **Fig. 4**) has a large impact on the steam consumption and exergy losses. Those exergy losses at the concentrator and the recycle cooler drastically increase with increasing liquid-to-solid ratio. **Fig. 5 a**), **5 a**), and **Fig. 8 a**) show an elevated cooling water consumption due to a high demand at the recycle cooler (see **Fig. 2** lower recycle loop) if the evaporated water in the concentration step has to be cooled with water. However, a concentration step with water only instead of a mixture with ethanol, could for instance be substituted with membrane technologies to pre-concentrate the isoflavones and recycle the permeate as solvent for the extraction, avoiding the evaporation and cooling of a large amount of water. This would lead to even less exergy input and losses, and would make water the preferred process option even more strongly (e.g. **Fig. 8** and **Fig. 11**).

In most cases, it seems that a large difference in sustainability can only be made by using water as a solvent. At the same time, this will probably require further purification of the extract, since the yield and purity of that extract is not as high as with ethanol [12]. Previous research has shown that water extracts about half of the isoflavones that can be extracted with 70% ethanol. However, this yield could be increased with an increased liquid-to-solid ratio or a well-designed counter-current process. Ethanol has a chemical exergy approximately 30 times higher than that estimated for the isoflavones. This points to a general rule for sustainable process design, where in an extraction process a “sustainability penalty” arises naturally on the misuse of solvents with chemical exergies too large compared with the target component. Therefore, given equal affinities between solvents and the to-be-extracted molecules, a design choice can be made between using large amounts of a solvent with low chemical exergy (where some losses can be tolerated), or using minimum quantities of solvents with higher chemical exergies (where losses should be kept to a strict minimum).

It is not surprising that extraction with water was found to be the more environmentally friendly option. As already stated by Dunn et al. [36], solvents tend to account for the majority of energy costs and greenhouse gas emissions. Process synthesis methodologies coupled with exergy analysis therefore help to reveal options that may not be considered in

traditional process development. Inclusion of exergy analysis particularly supports to achieve sustainable process synthesis as shown in this case.

The production of high-purity isoflavone isolates from a water or ethanol/water extract involves a further purification step. The demand for such a pure product depends obviously on the target application. It is not expected that inclusion of such purification steps in the analysis would affect the overall argument on the choice of solvents or sustainability of the process, due to the small resulting mass of the extract. Nevertheless, to include the purification step in a model will support the conceptual design of the total process. There are indications that solubilisation of the isoflavones in water may even be beneficial for further purification in case the latter is based on affinity separation [37].

Valorisation of the residue (**Fig. 7** and **Fig. 8**) helps redistributing the impact of cooling water and energy consumption, leading to higher sustainability and with even stronger indication of the advantages of water over ethanol. Of course, other considerations are important as well. The ethanol makes the product more microbiologically stable, but a separated residue without ethanol may be used directly as animal feed or for biogas production without further treatment needed to remove the ethanol residue.

A difference between exergy analysis in process optimisation and process conceptualisation is the significance of the exergy input. For process optimisation, the exergy loss is of importance mostly to pinpoint where improvement can be made. In the case of process conceptualisation, the exergy input can be compared for the different scenarios, and in our case shows that there is not much difference for a low liquid-to-solid ratio along the ethanol concentrations (**Fig. 5**). The curve increases slightly for low ethanol concentrations in most cases. If the head product of the distillation is lower than 45% ethanol the distillation can be carried out without a column, and the advantage of using a minimum number of theoretical plates and reflux is lost. In the cases shown in **Fig. 5** and **Fig. 7**, the ethanol is distilled to 95%, and then needs to be mixed again with water to achieve a concentration of for instance 10% ethanol, which results in a loss of exergy due to mixing of two different streams. In **Fig. 6** and **Fig. 8**, the distillation column was operated to minimise its energy use. Nevertheless, for low ethanol concentrations the overall system is still not very efficient compared to other scenarios.

At increasing liquid-to-solid ratios, the difference between the pure-water configuration and ethanol configurations becomes more evident, where the water configuration always has a lower exergy input than any ethanol configuration. A combination of low exergy input and low exergy loss should be the aim to find the most sustainable process option [38], which in

this case study corresponds to the use of water as the solvent for the extraction. An exergetic analysis helps to understand a system from an environmental/efficiency point of view better than energy or mass balances separately, and should be applied in combination with other constraints in green engineering and process synthesis [20]. The consideration of solely footprinting methods, such as a mass balance for water consumption or an energy analysis, might easily lead to higher local efficiencies at specific unit operations that then have the opposite effect on the performance of the total processing scheme, or that might complicate further optimisations. For example, if the focus is only on energy savings, heat integration schemes such as pinch analysis will deliver a reduction of the energy consumption. However, the more heat integrated the production site is, the less flexible it becomes to a change of flow rates and temperatures of the streams, or to the exchange of one unit operation by another. This can lead to overlooking possibilities where improvement of bigger impact can be made, or where alternative technologies can be introduced.

In the presented case, this would correspond to the cooling duty required in the recycle cooler of **Fig. 4**. Following a heat integration scheme one might be tempted, for example, to use the available heat in the steam of the evaporator as the utility to preheat the stream, reducing the consumption of steam and of cooling water. However, the exergy analysis (and its graphical representation in Grassmann diagrams) traces the actual cause of the inefficiency back to the concentration step, in which larger improvement can be achieved by using a non-thermal dewatering process such as a membrane separation. By elucidating the type, location and amount of exergetic wastes and losses, the combined analysis locates where a total process is inefficient or where improvements can be made.

The model used here does not directly integrate the process economics. However, an estimation can be made, considering that industrial ethanol is 400 times more expensive than water. The reduction of ethanol or even total elimination of ethanol is indeed also desired from the economic point of view, which supports our conclusions.

The type of (sustainability) analysis presented in our work should be executed in an early stage of the process synthesis to give directions for bench-scale experiments and the business case. This should prevent a too early fixation of a flow sheet to avoid costly or irreversible mistakes. With this analysis, we can pinpoint what the largest drawbacks of the extraction process are in an early stage and then work out the most efficient and sustainable process option considering state of the art or almost mature technologies if necessary.

CONCLUSIONS

The aim of the present study was to compare several process scenarios to extract isoflavones from okara. Within the framework of process synthesis, mass flow, energy, and exergy analysis were chosen to evaluate the efficiency of the system to facilitate the conceptualisation of a process to separate isoflavones from okara.

A dryer unit has less influence on the energy requirements and exergy losses than the amount of ethanol used. In most cases, and independently of whether the solids in okara are further valorised, water is the most energy and exergy efficient solvent.

Based on basic thermodynamic equations, analysis including exergy can reduce the complexity of process synthesis objectives combining several sustainability indicators (thus, objective or constraint functions) at the same time into one single objective indicator.

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Appendix A: Exergy calculations

The exergy flow through a process or unit operation can be described by:

$$B_{in} = B_{out} + B_{lost} \quad \text{Eq. (A.1)}$$

where

$$B_{lost} > 0 \wedge B_{lost} = B_{wasted} + B_{destroyed} \quad \text{Eq. (A.2)}$$

For simplicity, this work did not consider the exergies of potential energies or mechanical work.

Therefore, the total exergy of a stream equals the sum of its physical and chemical exergy:

$$B = B_{Ph} + B_{Ch} \quad \text{Eq. (A.3)}$$

where

$$B_{Ph} = B_T + B_P \quad \text{Eq. (A.4)}$$

$$B_T = \phi \cdot c_p \cdot \left[(T - T_0) - T_0 \times \ln \frac{T}{T_0} \right] \quad \text{Eq. (A.5)}$$

$$B_P = \phi \cdot \frac{R \cdot T_0}{M} \cdot \ln \frac{P}{P_0} \quad \text{Eq. (A.6)}$$

and

$$B_{Ch} = B_{St} + B_{Mix} \quad \text{Eq. (A.7)}$$

$$B_{St} = \phi \cdot \sum_1^n \frac{x_i b_i^0}{M_i} \quad \text{Eq. (A.8)}$$

$$B_{Mix} = \phi \cdot \frac{R \cdot T_0}{M} \sum_i^n x_i \cdot \ln x_i \quad \text{Eq. (A.9)}$$

B_T : thermal exergy of the stream [W]

B_P : pressure exergy of the stream [W]

B_{Ch} : chemical exergy of the stream [W]

B_{St} : standard chemical exergy of the stream [W]

B_{Mix} : exergy of mixing of the stream [W]

ϕ : stream mass flow [kg/s]

T_0 : reference temperature [K]

P_0 : reference pressure [Pa]

T : temperature [K]

P : pressure [Pa]

R : ideal gas constant [J/mol·K]

c_p : heat capacity of the stream [J/kg·K]

M : average molar mass of the stream [kg/mol]

M_i : molar mass of component i [kg/mol]

x_i : molar fraction of the component i [mol/mol]

b_i^0 : standard chemical exergy of component i [J/mol]

Appendix B: Model assumptions and other data

Other important assumptions and data not specified in the section Materials & Methods:

- Reference (environment) conditions are 293.15 K and 101.325 kPa.
- The process is continuous and in steady state, all unit operations behave ideally (adiabatic).
- Heat capacities of the different streams are constant over the temperature ranges considered.
- The heat capacities of okara and its related streams were estimated from their proximal composition following the method from Karel and Lund [39] for food materials.
- The heat capacity of isoflavones was estimated to be in average 1.02 kJ/kg·K using ChemBioDraw Ultra (v12.0.2.10.67, PerkinElmer).
- The chemical exergy of the solids of okara was estimated from its proximal composition, considering a chemical exergy of carbohydrates equal to 17.64 MJ/kg, of proteins equal to 22.61 MJ/kg, and of lipids equal to 43.09 MJ/kg. Those values were, in turn, derived from the estimation proposed by Szargut [33] for the chemical exergy of organic fuels.
- The raw material okara has an initial moisture content of 81% and a temperature of 373.15 K.
- The temperature required for extraction is set to 313.15 K.
- Heat exchangers are ideal. Temperatures of hot and cold streams are set to ensure a minimum temperature difference of 10 K for heat transfer. Heating duty is provided by steam (at 403.15 K, 270.26 kPa) and cooling duty is provided by cooling water (at 273.15 K).
- The solvent concentration of the spent okara is set to 50%, independent of the solvent used.
- Waste streams (condensate, vapour, hot cooling water, and spent okara if applicable) are emitted to the environment without additional processing.
- Solvent water and ethanol (concentration 95%) are available at 313.15 K.
- The mixture isoflavones/ethanol/water was considered to be ethanol and water only for the distillation.
- The bottom product of the distillation is set to 3% ethanol, which cannot be recovered for recycling.
- Ethanol-water equilibria data was obtained from Beebe et al. [40] and Perry et al. [41].

- Distillation calculations followed the McCabe-Thiele method. For simplification the column was considered to be adiabatic, the heat of mixing of the mixture water/ethanol was assumed to be zero, the molar latent heat of vaporisation of all water/ethanol mixtures was considered to be constant, and a constant molar overflow was assumed in the column. The feed stream was heated to its bubble point before entering the column. Under those assumptions, the minimum reflux ratio was determined and the reflux was set to 1.5 times the determined minimum reflux, which has been cited as a good estimate for the upper limit of the range for optimum reflux ratio for minimum column cost [42]. Other elements, such as sizing of the column, were not considered in this analysis.

Table B.1. Feed and distillate composition when column is operated for minimum energy use (e.g. Fig. 6 and Fig. 8)

Composition [% ethanol]	
Feed	Distillate
10	45
15	55
20	65
25	70
30	70
35	75
40	75
45	75
50	75
55	80
60	75
65	80
70	80
75	80
80	85
90	95

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Chapter 4

Isoflavone extraction from okara using water as extractant

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Abstract

We here report on the use of water as a ‘green’ extraction solvent for the isolation of isoflavones from okara, a by-product of soymilk production. At a low liquid-to-solid ratio of 20 to 1 and 20 °C, 47% of the isoflavones that can be extracted with 70% aqueous ethanol were extracted. The malonyl-glucosides were fully recovered with a ratio of 20 to 1, while β -glucosides were recovered with an increased liquid-to-solid ratio of 40 to 1. The extraction of aglycones was better at higher ratios, but levelled off before reaching a 100% yield. Temperature hardly affected the total amount of isoflavones. At a 20 to 1 ratio, 20 °C, and pH 10, there was no significant difference ($p > 0.05$) between isoflavone extraction in water and in 70% aqueous ethanol. The results suggest that water may be used as a green alternative for separation of isoflavones from okara.

INTRODUCTION

Okara is the by-product of soymilk and tofu production. Okara can be used in traditional dishes immediately after production, but when produced in large quantities on industrial scale, the by-product becomes a waste stream, usually distributed to farmers or incinerated [1, 2]. One peculiar characteristic of okara is its high moisture content of about 80%, which makes it very susceptible to spoilage, and which seems to be the largest challenge for economically attractive utilisation of this by-product. One route is therefore to dry the okara to allow storage and transportation to a site at which it can be further processed. However, okara is known to be difficult to dry [3], and research has increased in recent years to improve the efficiency of its drying without damaging the okara and its components [4, 5]. Drying is not only energy intensive; it also degrades the okara due to the heat load, which reduces its economic value. Another approach is therefore to bypass the drying and immediately process okara in the wet state for the recovery of valuable components [6]. The use of okara without pre-processing would preserve isoflavones, which are sensitive, thermally labile bioactive components. Isoflavones belong to a group of polyphenols believed to be partially responsible for the health benefits of soy [7, 8], and the recovery of bioactive components from by-products has become a research area of great interest [9]. Twelve isoflavones have been isolated from soybean, which can be classified in four main groups, namely the aglycones (daidzein, genistein, and glycitein), the β -glucosides (daidzin, genistin, and glycitin), the malonyl-glucosides (malonyl-daidzin, malonyl-genistin, and malonyl-glycitin), and the acetyl-glucosides (acetyl-daidzin, acetyl-genistin, and acetyl-glycitin) [10].

The total isoflavone concentration and the different isoflavone forms present in the soybeans and their products (including okara) depend on the soy variety, its cultivation, the process and the storage conditions [11-14]. The most common chemical changes in the isoflavone form include the decarboxylation of the malonyl to acetyl-glucosides and the ester hydrolysis of the acetyl or malonyl-glucosides to β -glucosides. Furthermore, cleavage of the glucosidic bond leads to an increased amount of aglycones [14, 15]. The soaking times and temperatures applied during processing lead to increased amounts of aglycones and glucosides in the according soy system [14, 16]. Cold-grinding instead of hot-grinding of the slurry during soymilk production also leads to an increased amount of aglycones due to prolonged β -glucosidase activity [17].

The isoflavone production process usually comprises at least the two steps of extraction and purification. The extraction step often uses a large amount of organic solvents, while the purification step involves multiple chromatography columns. Therefore, there is a clear

demand for alternative, more efficient and environmentally friendly ways to produce isoflavones. Aqueous ethanol is in general considered a good solvent for extraction of isoflavones from various sources [18-20]. Also for okara, a wide range of aqueous ethanol was tested with the result of 50-70% ethanol being superior amongst the solvents tested regarding yield and purity of isoflavones in the extract [6]. The use of high ethanol concentrations in the solvent may require either drying as a pre-treatment of okara to avoid dilution of the ethanol, especially if an industrially interesting low liquid-to-solid ratio is desired, or an extremely large amount of ethanol. Drying as well as distillation are both undesired operations as they are energy intensive and expensive. That is why water was tested as an alternative solvent for isoflavone extraction. In the scope of green separation technologies in modern food processing, aqueous extractions are considered in many cases more favourable in terms of energy and cost than any organic solvent extraction. A further reason to test the potential of water as solvent was the existence of isoflavones in okara in glucosylated forms, whose nature suggests good solubility in water. However, the 12 different structures of isoflavones in soy may generate difficulties since some are relatively apolar. Nevertheless, it can be a good solvent to recover the naturally present forms from the okara, leaving the altered forms in the okara. Previous studies show the potential of using hot, pressurised water or alkaline extraction for the extraction of flavonoids, but those results cannot be directly translated to soy, because the optimal extraction method and solvent largely depend on the target compounds and the surrounding matrix. Polyphenols include more than 4000 flavonoids with their solubility depending on their natural structure [21, 22]. In case of isoflavones from soy, it is known that some of the soy isoflavones are rather instable in their natural state, and will be affected by high temperatures, high pressures and long extraction procedures. Matrix components of okara such as proteins complicate the targeted extraction of isoflavones, especially when using water as solvent [6]. The fact that other solvents, such as methanol and ethanol allow good extraction [18] could explain why only very few studies were conducted using water as solvent for soy isoflavones [20, 23]. Therefore, the systematic investigation of different processing parameters on the isoflavones and their isolation in a water environment is indispensable for a basis of new environmentally friendlier ways to process isoflavones.

The selection of the solvent is part of the overall process design, which can be done based on the partitioning of the isoflavones over the okara and the extraction phase, which can be estimated experimentally, by matching the octanol-water partition coefficient ($\log P$) or by estimating the solubility parameters, based on models such as the UNIFAC/UNIQUAC model [24]. The extraction does not only depend on the equilibrium solubility between the matrix

and the solvent, but also depends on the nature of the binding in and accessibility of the matrix. Therefore we combine the solubility criteria with experimental extraction trials to find alternative process routes for the recovery of isoflavones from the by-product okara.

The solubility of isoflavones in a solvent depends on many different factors. There is the structure of the solute itself, which determines its polar/non-polar nature, hydrophobicity and tendency to form hydrogen bonds, but also parameters such as temperature, pH, and liquid-to-solid ratios influence the solubility of isoflavones in a solvent. Furthermore, the behaviour of isoflavones towards solvents can vary depending on the matrix they have to be extracted from [25, 26].

Up to date, there is no data available on the behaviour of isoflavones in the matrix of okara, which after processing occurs with rather high water content. Elucidating the specific reactions and mechanisms occurring in this system will help to retain the most natural profile of the isoflavones during processing or to obtain the isoflavone profile that is wanted in the product, and support the development of a milder and more environmentally friendly process to recover isoflavones from okara. The objective of this study is therefore to investigate the potential of water as extraction solvent for the recovery of isoflavones from okara. Results were compared to an extraction with 70% ethanol, and different parameters tested in order to discuss their effect on the yield and profile of an isoflavone extract.

MATERIALS AND METHODS

Materials

Ethanol (Sigma Aldrich Co., Schnelldorf, Germany) and Milli-Q water (Q-Gard 2 Purification Pack, Millipore, France) were used for extraction. Methanol and formic acid for HPLC analysis were purchased from Sigma Aldrich Co. (Schnelldorf, Germany) and acetonitrile from Biosolve B.V. (Valkenswaard, The Netherlands). Isoflavone standards: daidzin, glycitin, genistin, daidzein, glycitein, genistein, acetyl-daidzin, acetyl-glycitin, acetyl-genistin, malonyl-daidzin, malonyl-glycitin, and malonyl-genistin were purchased from Nacalai USA Inc. (San Diego, USA). The standards were dissolved in analytical grade DMSO (Sigma Aldrich Co., Schnelldorf, Germany). Solutions containing all 12 isoflavones standards with concentrations of 0.05–100 µg/g were prepared using methanol and stored at –20 °C. All solvents were HPLC grade.

Okara production

The okara was produced with an ASC50 soymilk system (ProSoya Inc.; Ottawa, Canada). Soybeans were soaked for 2h to soften the beans before adding the mixture into a cooker grinder vessel in a ratio 7:1 (14l water:2kg soybeans). After grinding, the slurry was heated up by steam injection and cooked for 3 min at 105 °C. Before the separation of okara from the soymilk, the slurry passed through a deodoriser vessel to remove undesired volatiles responsible for the beany flavour of the soymilk. At the outlet, the soy slurry had a temperature around 80 °C. A filter centrifuge separated the fibrous by-product okara from the soymilk. The mass balance of this process is described by Equation (1). The okara was stored at -20 °C in sealed containers until analysed.

$$\text{Soybeans (2 kg)} + \text{Water (14 kg)} + \text{Steam (7.2 kg)} = \text{Soymilk (20.2 kg)} + \text{Okara (3 kg)} \quad (1)$$

Moisture content

The moisture content of okara was analysed by heating the samples for 60 min at 130 °C. The dry matter was determined in order to calculate the concentrations of isoflavones on dry weight basis. Nevertheless, the isoflavones were extracted from the wet okara. The water present in the okara was taken into account when the total amount of the extraction solvent was identified.

Ethanol extraction

For the ethanol based extraction, pure ethanol was added in a tube containing wet okara until a ratio of 20 to 1 on dry basis was obtained. A final ethanol concentration of 70% was achieved taking the water content of okara into account. The samples were placed in a shaking water bath (Grant OLS200, Grant Instruments Ltd.; Cambridge, UK). Shaking was adjusted at 140 rpm for 60 min at 20 °C.

Following the extraction process, the samples were decanted in an EMD Millipore Amicon 8400 (Massachusetts, USA). A Whatman filter paper no. 1 was placed at the bottom of the Amicon equipment and air pressure was applied. The samples were filtrated under 3 atm until no more extract passed the filter. Afterwards, the wet extracts were completely dried using a vacuum drier at 45 °C and 0.1 atm. All extractions were done in triplicate.

Water extractions as a function of solvent quantity

Water was added in tubes containing okara until the ratio of solvent to dry weight of okara was 20, 30, 40, 50, 60, 70, 80, 90 and 100 to 1, respectively. The samples were placed in a 140 rpm shaking water bath for 60 min at 20 °C and treated as described in the second paragraph of the section ‘ethanol extraction’.

Water extractions as a function of temperature

Water was added in tubes containing okara until 20:1 solvent to dry weight of okara ratio was achieved. The samples were placed in a 140 rpm shaking water bath for 60 min at 20, 35, 50, 65, 80 and 95 °C, respectively. Following the extraction process, samples were subjected to the treatment described in the second paragraph of the section ‘ethanol extraction’.

Water extraction as a function of pH

The pH of the samples was adjusted at 2, 3, 4, 5, 6, 7, 8, 9 and 10 using 1 M HCL and 1 M NaOH solutions. The solvent to dry weight ratio in the tubes was kept at 20:1. The samples were placed in a 140 rpm shaking water bath for 60 min at 20 °C and treated as mentioned in the second paragraph of the section ‘ethanol extraction’.

Protein analysis

The protein content of samples was analysed by Dumas. The nitrogen content of the samples was determined using a FlashEA 1112 NC analyser (Thermo Fisher Scientific Inc., MA, USA). Each sample was measured in triplicate and a conversion factor of 6.25 was used to calculate the protein content.

Isoflavone analysis

To exclude any differences due to the analytical measurement, and to obtain suitable isoflavone concentrations in the extract for analysis, the extracts were dried prior to analysis

[10]. A vacuum dryer ($T < 45^{\circ}\text{C}$) was used to be able to handle more samples at the same time. Extracts were dried completely, and weights of the dried extracts were recorded accurately for the calculation of the purity. After preliminary tests with different solvents, 50% methanol was chosen to dissolve the extracts for further HPLC analysis, since this mixture dissolved the extracts well, and isoflavone standards were also diluted in methanol. A certain percentage (50%) of water was necessary to dissolve the entire extract.

Prior to the HPLC analysis, the dissolved extracts were filtered through a $0.20\text{ }\mu\text{m}$ Whatman Spartan 13 syringe filter. A Dionex UltiMate 3000 Basic Automated HPLC system with a photodiode array detector (PDA) from Thermo Scientific (Massachusetts, USA) was used to determine the isoflavone content of the prepared HPLC samples. The temperatures of the autosampler and the column oven were set to 10 and 40°C , respectively. The column used was a $2.1 \times 150\text{ mm}$, $3\text{ }\mu\text{m}$, dC18 Atlantis column obtained from Waters Corporation (Massachusetts, USA). A gradient was composed of the following two eluents: 0.1% formic acid in Milli-Q water and 0.1% formic acid in acetonitrile. The injection volume was $3\text{ }\mu\text{l}$ and the flow rate was maintained at 0.3 ml/min . Standard solutions containing all 12 isoflavones in pure form were used for calibration and identification. The peak areas of the standard solutions and the extracts were measured using Dionex Chromeleon 7 Chromatography Data System, Thermo Scientific (Massachusetts, USA), at a wavelength of 254 nm .

The purity of isoflavones in the extract was expressed as the ratio of the mass of isoflavones to the mass of the total dry extract.

Statistical analysis

Data was analysed by analysis of variance (ANOVA) using IBM SPSS version 20 (SPSS Inc., Chicago, USA). Differences within the group were determined using Least Significant Difference (LSD) multiple comparison analysis. Differences at a $p\text{-value} < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Comparison of ethanol and water based extraction

The moisture content of okara was $79 \pm 1\%$, which was taken into account for the water and 70% aqueous ethanol extractions. The liquid-to-solid ratio was adjusted to 20:1 (based on 1g dry solids) for both extractions in order to obtain comparable results. The total isoflavone concentration thus obtained was $479 \pm 83 \mu\text{g/g}$ in the water extract, and 1018 ± 43 in the 70% aqueous ethanol extract (**Table 1**).

Table 1. Isoflavones present in okara, their elution order, and concentrations ($\mu\text{g/g}$) in the 70% aqueous ethanol extract and water extract.

Isoflavone	Elution order	70% aqueous ethanol	Water	
		($\mu\text{g/g}$) ^a	($\mu\text{g/g}$) ^a	(%) ^c
Daidzin	1	160 ± 7	108 ± 22	68
Glycitin	2	26 ± 2	19 ± 3	73
Genistin	3	171 ± 7	71 ± 16	42
Malonyl-daidzin	4	85 ± 1	85 ± 5	100
Malonyl-glycitin	5	15 ± 1	14 ± 2	93
Acetyl-daidzin		n.d. ^b	n.d. ^b	
Acetyl-glycitin		n.d. ^b	n.d. ^b	
Malonyl-genistin	6	169 ± 4	144 ± 21	85
Daidzein	7	173 ± 8	22 ± 10	13
Glycitein	8	19 ± 1	8 ± 1	42
Acetyl-genistin	9	6 ± 0	5 ± 0	83
Genistein	10	194 ± 14	3 ± 4	2
Total isoflavones		1018 ± 43	479 ± 83	

^a Data obtained from three replicates; ^b Not detected; ^c relative to the extraction with 70% aqueous ethanol.

Most soybean varieties contain naturally very little or no aglycones [10]. Processed soy products often contain an increased amount of aglycones due to processing steps involving heating, grinding, or soaking. This increase of aglycones can often be attributed to the hydrolysis of other forms such as malonyl-glucosides and glucosides to their respective aglycones [11-14, 27]. The okara used in this study contained a high amount of aglycones, which is probably caused by the cold-grinding step leading to increased β -glucosidase activity, but also the subsequent cooking of the slurry at 105°C for several minutes. In fact, studies where a high amount of aglycones was detected in the okara generally used okara

produced on laboratory scale. Okara produced on industrial scale, contained a more natural profile of isoflavones (predominantly of malonyl-glucosides and β -glucosides) closer to that in soybeans, [1, 28], likely due to lower temperatures and shorter contact. An additional reason for the high concentrations of aglycones in okara is their lower polarity compared with the other forms, which makes them remain in the residue during soymilk extraction and partition less into the water extract (the soymilk) [25]. The absence or low concentration of the acetyl-glucosides found in okara (**Table 1**) was in line with literature [1, 11, 16, 28]. Acetyl-daizidin and acetyl-glycitin were not identified at all in the samples, neither with water nor with aqueous ethanol. The concentration of acetyl-genistin was low compared to the concentrations of the rest of the isoflavones. Therefore, the group of acetyl-glucosides was monitored, but not analysed further.

Fig. 1 summarises each isoflavone group present in the aqueous and aqueous ethanol extract from okara. In the ethanol extract, the aglycones accounted for 38% of the total isoflavones, the β -glucosides for 35% and the malonyl-glucosides for 26%. In the water extract at the same solvent ratio (**Fig. 1A**), the aglycones accounted for 7% of the total isoflavones, the β -glucosides for 41% and the malonyl-glucosides for 51%. The malonyl and β -glucosides generally dissolved well in water (**Fig. 1**). However, also for the glucosides not the entire yield was reached, which either indicates saturation of the glucosides in water or interaction of those components with the matrix.

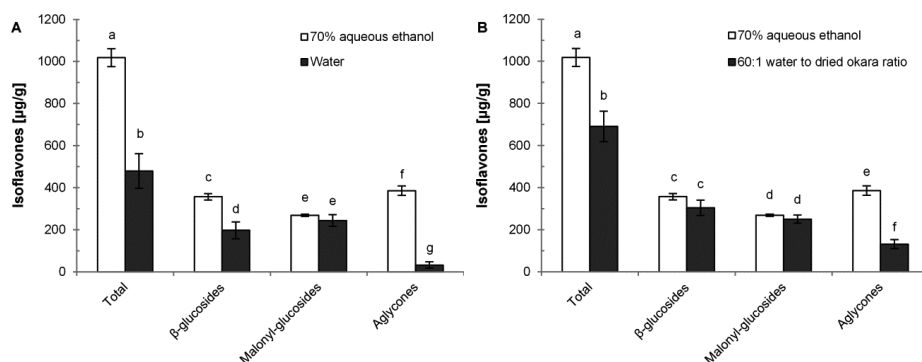


Fig. 1. Isoflavone concentrations ($\mu\text{g/g}$) obtained with 70% aqueous ethanol (white bars) and water only (grey bars). Extraction conditions were $T = 20^\circ\text{C}$, $t = 1\text{h}$, and (A) liquid-to-solid ratio = 20:1 (solvent to dry weight okara) for both solvents; (B) liquid-to-solid ratio = 20:1 for 70% aqueous ethanol, and liquid-to-solid ratio = 60:1 for water only. Bars with the same letter are not significantly different ($p > 0.05$).

The elution order of the isoflavones as seen in **Table 1** coincides with that reported by Wang et al. [10]. According to their elution order during HPLC analysis, the glucosides were expected to be even less hydrophobic than the malonyl-glucosides, as it was also speculated by Murphy, et al. [25]. Based on the hydrophobicity and polarity of those two isoflavone groups, water may perform equal or better than ethanol for the extraction of the β -glucosides. However, ethanol was proven to be a superior extraction solvent compared to water for the β -glucosides (**Fig. 1A**). Proteins have been suspected to have strong interaction with isoflavones [13, 29, 30], and one may expect that isoflavones would interact stronger with proteins in water, instead of in ethanol [31]. During the production of soymilk, the proteins and isoflavones are exposed to each other, and may form complexes or aggregates. Isoflavones are believed to interact with the soy proteins by hydrophobic bonding. Soy proteins are approximately 90% globular, with a hydrophobic interior and a hydrophilic surface [32]. Consequently, the associated isoflavones would be concealed from the aqueous phase [33]. This may be stronger for the proteins in okara, since they were not extracted in the aqueous phase during soymilk production, and thus must be more hydrophobic than the ones that were extracted. Generally, the more water soluble a component is, the less prone it is to interact with proteins, and polyphenol dissolution in a solvent is more favourable than complexation with proteins [31]. Therefore, the β -glucosides and even more the aglycones with a lower affinity to water than the malonyl-glucosides could associate more strongly with proteins when dissolved in water.

Table 1 indicates the percentages of each isoflavone solubilised in water compared to the amount solubilised in 70% ethanol. The genistin molecule of each form (genistein, genistin, malonyl-genistin), with its additional hydroxyl group on one of the aromatic rings, was always detected in lower amounts in the aqueous extract than the respective daidzin or glycitin. A somewhat different behaviour of genistein and its derivatives was observed by several authors [15, 30, 34], and demonstrates the complexity of polyphenolic compounds with their complex stereochemistry, and the difficulty of predicting their behaviour in different systems, even within a group of closely related molecules such as the twelve isoflavones occurring in soy. In general, the solubility increases with the number of hydroxyl groups [35], but the additional hydroxyl group of the genistin series also affects the degree of ionisation at a certain pH which will change the hydrophobicity of the molecule, and, therefore, the solubility in water.

While the overall isoflavone concentration in a solvent (**Fig. 1**) is an important parameter, the purity of the isoflavones in the extract is just as important. The percentage of isoflavones in the water extract was 0.6% and in the ethanol extract 1.9% based on the dry matter of the

extract. The selectivity of water for isoflavones was, therefore, three times less compared to ethanol, probably due to increased extraction of other components, such as proteins. Thus, using water may necessitate further purification of the extract.

The effect of solvent quantity on water based extractions

The total amount of isoflavones extracted as function of the water to okara ratio is shown in **Fig. 2A**. Up to a ratio of 50:1, the total isoflavone concentration increased indicating saturation of the components at lower ratios. After that, the concentration of isoflavones remained relatively stable around 700 µg/g. The different groups of isoflavones however showed differing behaviour. The concentration of the malonyl-glucoside group did not change with the liquid-to-solid ratio (**Fig. 1** and **Fig. 2B**). The malonyl-glucosides dissolved completely in water for a 20:1 liquid-to-solid ratio indicating that the system had not been saturated. Therefore, the increase in the total isoflavone concentration was attributed to the increase in the concentration of the β-glucosides and the aglycones (**Fig. 2B**).

The aglycones in particular showed a linear increase at ratios below 50:1, indicating that their extraction is limited by their low affinity with water. Indeed, the highest amount of aglycones obtained with higher water to solids ratios (approx. 150 µg/g) was only about one third compared to its respective for the ethanol extract (approx. 390 µg/g) (**Fig. 1B**).

The increase of the β-glucosides was not as pronounced as for the aglycones. However, when concentrations levelled off, a similar value as in the aqueous ethanol extract was found in the water extract (**Fig. 1B**). Water in high liquid-to-solid ratios could, therefore, be an equal extraction solvent to ethanol for both the β-glucosides and the malonyl-glucosides. These are the components naturally present in soybeans.

The partitioning of the isoflavone groups into water and okara solids can be expressed by the following mass balance:

$$M_s c_0 = M_s c_s + M_l c_l \quad (2)$$

where M_s is the mass of okara solids (dry), c_0 the concentration of each isoflavone group present in okara, c_s the concentration of isoflavones in the solids, M_l the mass of the liquid (water), and c_l the concentration of isoflavones in the liquid.

Assuming a constant partitioning coefficient k with

$$c_s = kc_l \quad (3)$$

and combining equation 2 and 3, a relationship for the extracted amount of isoflavones from 1 g of okara depending on the liquid-to-solid ratio can be made:

$$\frac{M_L c_L}{M_S} = \left(\frac{M_L}{M_S k + M_L} \right) c_0 = \left(\frac{1}{\frac{k}{\left(\frac{M_L}{M_S}\right)} + 1} \right) c_0 \quad (4)$$

The resulting fit is shown in **Fig. 2C**. The estimated model parameter values for k are 197, 21, 0, and for c_0 478, 404, 249, describing the extraction of the aglycones, β -glucosides, and malonyl-glucosides, respectively (equation 4). The high partitioning coefficient k for the aglycones ($k = 197$) indicates a high affinity for the matrix, $k = 21$ for the glucosides an intermediate affinity, and the low value of 0 for the malonyl-glucosides shows that the components are very well soluble, and have no discernible interaction with the matrix. The c_0 values for the isoflavone concentration of the malonyl-glucosides are very close to the experimental values found for the 70% ethanol extraction (**Table 1**). The concentration of the aglycones found with the ethanol extraction is lower than the model value, suggesting that also 70% ethanol is not capable of extracting all aglycones. The concentration of the glucosides was also lower than predicted with this model, but not deviating as much as the value for the aglycones. Nevertheless, this simple model seems useful to describe the extraction of the various isoflavone forms in water.

The concentrations of β -glucosides and aglycones were not changing above a certain liquid-to-solid ratio. The determination of that point allows the estimation of an isoflavone solubility limit in water in the presence of the okara matrix for each of those molecules, and they are presented in **Table 2**. Unfortunately, the solubility of the pure isoflavones in water has not been reported extensively. The most studied molecules of the isoflavones are genistein and daidzein, and their solubility in water (usually given in molar fractions) was recalculated to mg/L and shown in **Table 2** [36-38]. The reported solubility limits for daidzein, as well as for genistein are of similar order of magnitude as our values for the apparent solubility. Somewhat higher values of solubility limits of the pure components in pure water suggest that in those experiments no matrix material was present that hinders or binds part of the molecules. Also in watery soy whey (very diluted soy matrix) the solubility of genistin was found to be higher with approximately 20 mg/L at 20 °C [34].

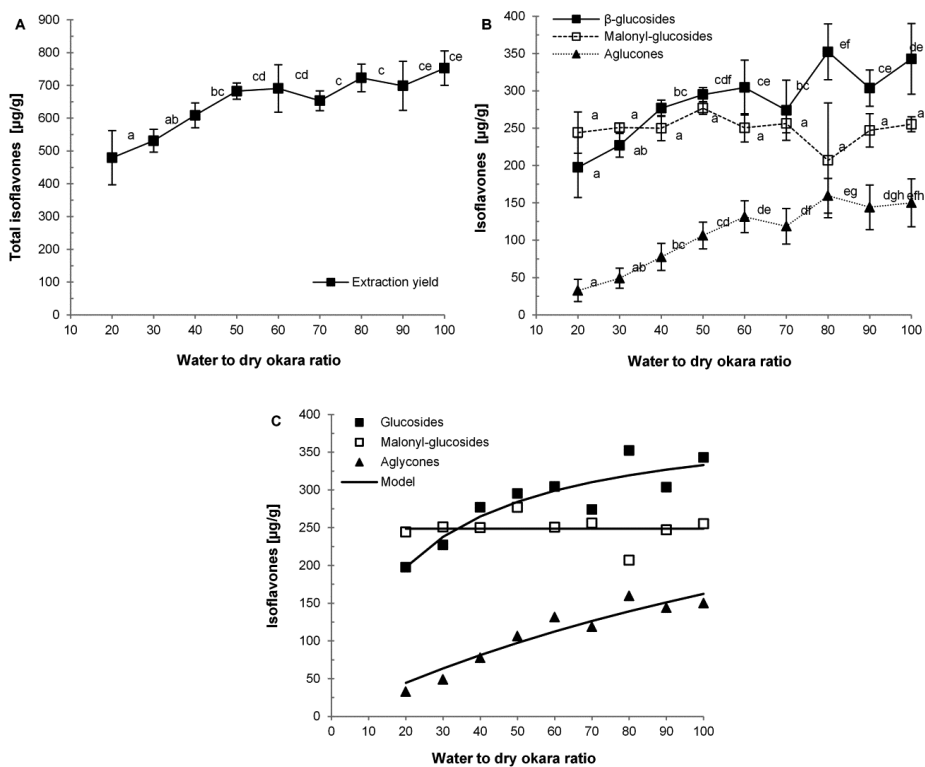


Fig. 2. Isoflavone concentrations (µg/g) obtained with different liquid-to-solid ratios ranging from 20 to 100 for (A) total isoflavones expressed as extraction yield, and for (B) β-glucosides, malonyl-glucosides, and aglycones. Extraction conditions were T = 20 °C and t = 1h. Data points of each curve with the same letter are not significantly different (p > 0.05). (C) Model lines derived from equation 3 using parameters of Table 2.

Table 2. Estimated isoflavone solubility limit in water within the okara matrix, and pure components in water for the β-glucosides and the aglycones at room temperature.

Isoflavone	Estimated isoflavone solubility limit within okara matrix (mg/L) ^a	Isoflavone solubility limit in water (mg/L)
Daidzin	3.04 ± 0.25	n.a. ^b
Genistin	2.30 ± 0.29	n.a. ^b
Glycitin	0.49 ± 0.02	n.a. ^b
Daidzein	1.19 ± 0.04	2.54 [37]
		0.86 [38]
Genistein	0.79 ± 0.28	0.81 [37]
		1.43 [36]
Glycitein	0.26 ± 0.02	n.a. ^b

^a Data obtained from three replicates; ^b No data available.

The effect of temperature on water based extraction

J. G. Wu, et al. [36] and Yang, et al. [38] reported that solubility of genistein and daidzein, respectively, increased with increasing temperature. Higher temperatures used during extraction may cause isoflavone degradation resulting in lower isoflavone concentrations or a different isoflavone profile [11, 27]. The ambiguous effect of temperature on the isoflavone extractability from okara was therefore tested in this study at a liquid-to-solid ratio of 20 to 1.

To determine the effect of temperature on the changes of one type of isoflavone, each isoflavone was studied, but the end result could be summarised in isoflavone groups as shown in **Fig. 3** since the trend of molecules of the same group was the same. Statistical analysis showed no difference in the total isoflavone concentrations obtained at temperatures from 20 to 95 °C. However, there was a significant decrease in the concentration of the malonyl-glucosides at 65 °C and above. The glucoside group did not show a significant change, but a trend towards an increase can be seen, which may be attributed to the hydrolysis of the malonyl forms, and not to an increase in solubility, as was discussed in a patent from Gugger et al. [34]. Looking at each glucoside separately, a significant increase was noticed with a concurrent decrease of the corresponding malonyl glucoside. Again, this effect loses significance by grouping the isoflavone forms and looking at the total isoflavone content (**Fig. 3**). Temperatures between 20 and 50 °C lead to an increase of the aglycones ($31 \pm 12 \mu\text{g/g}$ to $72 \pm 21 \mu\text{g/g}$) as shown in **Fig. 3**. However, the increase of aglycones was too small to lead to a noticeable increase of the total amount. Furthermore, the aglycone concentration of $93 \pm 19 \mu\text{g/g}$ at 80 °C was still only a fourth compared to its respective amount obtained with ethanol as an extraction solvent ($386 \pm 22 \mu\text{g/g}$), confirming that the aglycones could interact with other okara components as mentioned previously. Okara and its isoflavones have already been subjected to high temperatures during processing of the soybeans into soymilk and okara, which might explain the fact that we find low sensitivities to the temperature, compared to other studies [20, 38].

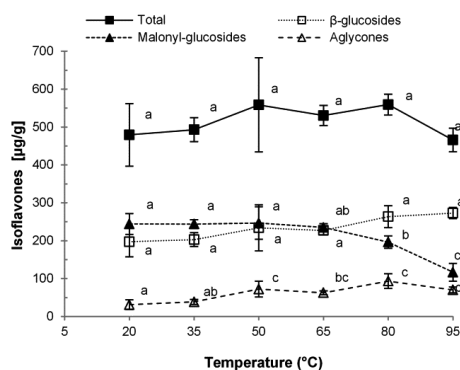


Fig. 3. Extraction yield ($\mu\text{g/g}$) obtained at different temperatures ranging from 20 °C to 95 °C for total isoflavones, β -glucosides, malonyl-glucosides, and aglycones. Extraction conditions were $t = 1\text{ h}$ and liquid-to-solid ratio = 20:1. Data points of each curve with the same letter are not significantly different ($p > 0.05$).

The effect of pH on water based extractions

A different pH in soy protein isolate production was reported to influence the hydrophobic interactions [29]. Therefore, the effect of the pH on the extractability of isoflavones from okara was analysed in the present study. **Figure 4A** shows both the total isoflavone concentration and the protein concentration after water extraction from okara at pH 2 to 10. The isoflavone and protein concentrations followed the same trend. The protein solubility curve concurs with recently published results by Vishwanathan, Singh, & Subramanian [39]. The combination of the protein content and isoflavone concentration in the okara extracts is however new, and is an indication of the association of isoflavones with proteins. Both had a minimum around pH 3 and 4, and increased monotonously to a maximum concentration of $911 \pm 34 \mu\text{g/g}$ for isoflavones and $416 \pm 29 \text{ mg/g}$ for proteins. The highest isoflavone concentration ($911 \mu\text{g/g}$) was obtained at pH 10 resulting in a percentage of 90% isoflavone recovery and no significant difference ($p > 0.05$) compared to the aqueous ethanol extraction. This result was in line with a study about the extraction of isoflavones from red clover, where the concentrations of isoflavones were also highest after extraction from red clover at pH 10 [40]. Nevertheless, the concentration of the malonyl-glucosides seemed to be slightly lower. A decrease in the malonyl-glucoside concentration above pH 9 was revealed by plotting the concentration of each isoflavone group as a function of pH (**Fig. 4B**). This effect could be attributed to the de-esterification of the carboxyl group of those isoflavones at alkaline conditions [29, 41]. In a study of Speroni, Milesi, & Anon [42], defatted soy flour was

dispersed in water and the pH adjusted [42]. At pH 11 there was a strong decrease of the malonyl-glucoside genistein, accompanied by an increase of the β -glucoside genistin. However, in our study an additional increase of aglycones and β -glucosides was found at pH 9 and 10, which could not only be attributed to the conversion of malonyl-glucosides to aglycones or β -glucosides, especially at pH 9. The changing solubility properties of isoflavones and proteins at different pH's could rather explain the different extraction results of isoflavones in the water environment.

One may argue that the solubilisation of the isoflavones may be due to the ionisation of the isoflavones themselves, or due to the proteins becoming more ionised (leading to less hydrophobicity and subsequently less interaction with the isoflavones), or even due to the increased solubilisation of the protein. It is possibly a combination of all three. On the one hand, the purity did not significantly change ($p > 0.05$) from pH 7 to pH 10, which confirms the corresponding increase of isoflavones with other solids and the proteins. On the other hand, protein precipitation after extraction at high pH, only leads to a fraction of isoflavones in the protein precipitate, which suggests that only a part of isoflavones, depending on processing conditions, is still strongly associated to the proteins [13, 30]. Then again, the minimum in extraction yield of the isoflavones coincides with the minimum for proteins, which indeed suggests a relation between the protein and isoflavone extraction. This would all point towards the reduction in the hydrophobic interaction with the proteins being the dominant mechanism to explain the increased isoflavone yields at high pH.

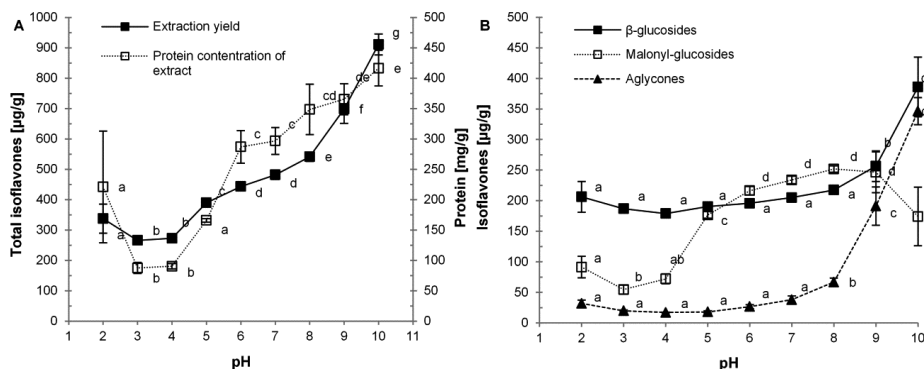


Fig. 4. (A) Extraction yield for total isoflavones (μg/g) and protein content in the extract (mg/g) and (B) extraction yield for β -glucosides, malonyl-glucosides, and aglycones, obtained at different pH ranging from 2 to 10. Extraction conditions were $T = 20^\circ\text{C}$, $t = 1\text{ h}$ and liquid-to-solid ratio = 20:1. Data points of each curve with the same letter are not significantly different ($p > 0.05$).

Water as a green extraction solvent

Since the protein content and the isoflavone content are coupled, one may assume that the protein – isoflavone interactions have a large effect on the extraction process. However, the solubility also plays an important role, shown for example by the different amounts extracted with different water-to-okara ratios. Therefore, the extractability of the isoflavones in the water environment is a combination of the affinity with the matrix (mainly proteins) and the solubility in water.

Several choices have to be made for the design of a process. If the most natural profile of isoflavones is desired in the end product, temperatures above 65 °C and a pH above 9 should be avoided since those extreme conditions convert the malonyl forms into their respective glucosides. For the simplest process without any addition of a base, with water only, the majority of the aglycones will not be extracted. Higher liquid-to-solid ratios are essential to recover all glucosides and malonyl glucosides compared to aqueous ethanol extraction. Higher ratios of water can be compensated for with modern, more energy efficient processes for water recovery, for example through reverse osmosis and the application of counter-current process flows.

The composition and therefore the extractability of the isoflavones strongly depend on the processing conditions used during soymilk production. Milder processing during soymilk extraction, by using lower temperatures and/or shorter processing times, will preserve the natural profile of isoflavones better; it will lead to less aglycones. Theoretically, the same yield as in an ethanol extract could then be attained if only those isoflavone groups are present.

Full recovery of isoflavones was achieved by adjustment of the pH even at lower liquid-to-solid ratios, which also included the aglycones, believed to be the most bioavailable form of all. To summarise, varying affinities of the isoflavones to the matrix in comparison to water largely influences the selection of extraction conditions. That is why one should give more importance to the different groups of isoflavones present in a material.

In the view of sustainable process synthesis, the water extract has the advantage that it can be used as is for further purification by chromatography, in which it may facilitate the adsorption [43]. Further, one should bear in mind that the purity is lower in the water extract, as some proteins are co-solubilised. Therefore, it is worth investigating whether the purity of the extract has a large impact on the efficiency of the subsequently used purification method. Aside from chromatographic methods, other concentration methods such as ultrafiltration

may be applied if all isoflavones are present in the water extract. Thus, detailed knowledge of the okara – water system is essential for further development of efficient processes for the valorisation of the by-product okara.

CONCLUSIONS

The feasibility of water as a green alternative for the extraction of isoflavones from okara has been evaluated. Malonyl-glucosides were extracted efficiently even with low amounts of water, while the β -glucosides solubilised well in water at larger liquid-to-solid ratios. The aglycones in general dissolved only partially in water, even at higher liquid-to-solid ratios.

The extraction temperature did not have a large influence on the extraction yield. Only a slight increase was obtained for the aglycones with a temperature increase from 20 °C to 50 °C. Above 50 °C the aglycone yield remained unaffected, but the yield of the most unstable malonyl forms began to decrease. The more apolar aglycones could be solubilised in water by ionisation. At pH 10, 90% of the yield relative to an extraction with 70% ethanol was achieved. The purity of the extract was around 0.6% of isoflavones in the dried water extract and around 2% for the dried ethanol extract.

The overall behaviour indicated that the interactions between proteins and isoflavones probably play an important role, next to the solubility in water. The findings in this study show that primary separation of isoflavones can be achieved with water. The underlying mechanisms of water extraction have been revealed which facilitate the development of a sustainable and economic process for utilisation of the by-product okara.

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Chapter 5

Adsorption of isoflavones on PVPP in the presence of a soy matrix

This chapter is submitted for publication as Jankowiak, L., van Avermaete, I., Boom, R.M., & van der Goot, A.J. Adsorption of isoflavones on PVPP in the presence of a soy matrix.

Abstract

The adsorption of isoflavones from an okara extract onto PVPP was studied for a pH ranging from 4 to 7.

In general, the affinity of aglycones to PVPP was around 10 times higher than the affinity of the glycosides. Towards lower pH, the adsorption performance improved for the glycosides, especially for the malonyl-glucosides, but not for the aglycones. Towards neutral pH the affinity also increased, and showed the highest affinity for the total amount of isoflavones. Due to a large amount of protein present in the extract, some isoflavones were lost at lower pH due to the precipitation of protein, which led to losses of 3% - 25% in the protein fraction without any addition of PVPP.

Moreover, PVPP was added to the crude starting material (i.e. okara), investigating the effect of the matrix on the adsorption and the possibility to combine the extraction and adsorption process. The isoflavone extraction was modelled using affinity constants of isoflavones to the matrix and the PVPP. The simulation showed that concurrent PVPP addition results in more extraction from the isoflavones from the matrix.

INTRODUCTION

Okara is a by-product from the soymilk producing industry, and contains a large amount of fibre (~50% d.w.), around 30% of protein, and 15% fat. Amongst the minor components that okara contains are isoflavones. Isoflavones belong to a large group of polyphenols, which possess a high value for pharmaceutical and food applications. The isolation of those polyphenolic components would create value from the by-product, and reduce the remaining waste. However, conventional separation methods are not efficient enough to make the utilisation of those components worthwhile.

Okara poses several challenges for efficient and environmentally friendly utilisation, which are discussed by Jankowiak *et al.* [1]. While isoflavones can be extracted with solvents, such as ethanol, we investigated the use of water as an alternative that is more environmentally benign. We therefore investigated the behaviour of the isoflavones in an aqueous extraction phase, and their interaction with other components in the okara matrix [2]. The glycosidic isoflavones are better available in the water phase than the aglycones, which can nevertheless be captured in an aqueous phase at alkaline pH since they dissociate at higher pH's. One of the complications of using water as extractant is the fact that okara swells considerably in water, and, along with the isoflavones, impurities are therefore present in the water phase. Suspended proteins are not the only impurity in this extract. Soluble carbohydrates make up another major part of the components. Therefore, the isoflavones have to be separated more selectively from the resulting solution/suspension, and we chose to have a second step to obtain a purer extract by using selective adsorption of the isoflavones on polyvinyl pyrrolidone.

Cross-linked polyvinyl pyrrolidone or polyvinyl polypyrrolidone (PVPP) finds wide application in the food industry for clarification and stabilisation of juices, wine, beer, and other beverages. In its cross-linked form, this polymer is water-insoluble, and has the ability to bind polyphenolic components mainly based on hydrogen bonds and hydrophobic interaction. For the formation of hydrogen bonds, the phenolic molecule should be present in its protonated form [3, 4]. This can be the case at neutral pH, but PVPP is often used at lower pH to ensure protonation of the adsorbate. Not only the state of protonation of the adsorbate is important; the number of phenolic hydroxyl groups available for hydrogen bonding seems to play a large role as well [4, 5].

The use of PVPP has mainly been targeted to the removal of unwanted polyphenols that lead to haze formation, bitterness or astringency, or instability in a beverage, and easy regeneration

of PVPP made it suitable for large-scale applications. The potential of the large resulting side stream from the beer production has only recently been investigated as a source of polyphenols [6, 7]. The interest in those polyphenols is threefold: their consumption is encouraged for a range of suspected health benefits [8], and they often display high antioxidant activity, which makes them suitable as a natural replacement for synthetic antioxidants often used in the food industry. Finally but equally important, turning a waste stream into a valuable product reduces the use of other valuable resources, and thus the amount of waste generated.

The reported mechanisms for phenolic components to bind to PVPP are often investigated in pure model systems (i.e. the pure polyphenol and PVPP in a solvent) [3, 5], or with the purpose of using PVPP as an adsorbent for analytical assays [4]. However, adsorption may take place in a different manner if components are in a complex solid-liquid system [9, 10]. As an example, the interaction between polyphenols and proteins may interfere with the interactions between the polyphenols and PVPP [11], and the presence of a food matrix may affect the specific behaviour of the desired components in that matrix and their adsorption behaviour [10, 12]. Furthermore, the structure of the polyphenol largely affects its affinity to the adsorbent [5]. The distinctive behaviour of the different isoflavone groups present in the okara extract was therefore investigated at different pH values. The results were interpreted using affinity constants to the matrix and the PVPP to support the development of an efficient and more sustainable separation process for isoflavones from the by-product okara.

MATERIALS AND METHODS

Alkaline extract production

Okara was produced as described by Jankowiak et al. [2] with an ASC50 soymilk system (ProSoya Inc., Ottawa, Canada). The produced okara had a moisture content of $79 \pm 0.5\%$.

Crude extracts were produced by adding water to 20-40 g of crude okara until a solvent-to-solid ratio of 20:1 on dry basis was obtained. The pH was adjusted to pH 10 with 1M NaOH. The samples were placed in a shaking water bath and extraction was done at room temperature for 1h. Then, the samples were decanted in an EMD Millipore Amicon 8400 (Massachusetts, USA). A Whatman filter paper no. 1 was placed at the bottom of the Amicon equipment and air pressure was applied. The samples were filtered until no more extract passed the filter.

Protein precipitation

Protein in the alkaline extract was precipitated with 1M HCl. The pH was adjusted to 4, 4.5, 5, 5.5, 6 and 7, respectively. After 20 min, the samples were centrifuged at 4700g for 20 min in a Sorvall Legend XFR Centrifuge (Thermo Scientific, Waltham, USA). The supernatant was separated and isoflavone content measured.

Adsorption of isoflavones with PVPP

PVPP (110 μm) was purchased from Sigma-Aldrich Co. (Schnelldorf, Germany) and was added to Milli-Q water to prepare a 10% w/v slurry. The slurry was hydrated for at least 120 min before usage. Samples of 12 mL alkaline extract were placed in plastic tubes and the pH was adjusted to values of 4, 4.5, 5, 5.5, 6 and 7, respectively, using 1M HCl. For each pH value, the PVPP slurry was added in amounts ranging from 2 to 20 mg PVPP/mL extract under constant stirring of the slurry and the samples. All samples were stirred for 20 min at room temperature and subsequently centrifuged at 4700g for 20 minutes in a Sorvall Legend XFR Centrifuge (Thermo Scientific, Waltham, USA).

Adsorption of isoflavones to PVPP within the okara matrix

PVPP was added to Milli-Q water to prepare a 10% w/v slurry. The slurry was hydrated for at least 120 min before usage. A sample of 40 g wet okara was extracted for 1h using floating pH (\sim pH7). The water was added to the okara until a ratio of 20g water to 1g dry okara solids was reached. After extraction, the fibrous solids were not filtered from the liquid. The PVPP slurry was gradually added to the okara-water mixture in amounts ranging from 2 to 20 mg PVPP/mL extract, and under constant stirring of the slurry and the sample. After each addition of PVPP, the material was mixed at room temperature for 20 min and subsequently centrifuged at 4700g for 20 min in a Sorvall Legend XFR Centrifuge (Thermo Scientific, Waltham, USA). A sample of each supernatant was recovered for isoflavone quantification.

Determination of protein and total sugar content

The protein content was analysed by the Dumas method. The nitrogen content of the samples was determined using a FlashEA 1112 NC analyser (Thermo Fisher Scientific Inc., MA, USA). A conversion factor of 6.25 was used to calculate the protein content.

The total sugar content was estimated by a modified Phenolic-Sulphuric Acid method according to Chow and Landhäusser [13]. A 1:1:1 glucose:fructose:galactose solution was used as a standard. 0.5 mL of extract was combined with 1 mL phenol 2% w/v and 2.5 mL concentrated sulphuric acid (95-98%). Samples were stirred in a vortex at 3000 rpm for 10 seconds and left to cool down at ambient temperature for 30 min. Absorbance was read at 490 nm using a Beckman Coulter UV/VIS Spectrophotometer DU 720 (Pasadena, USA).

Isoflavone analysis

Isoflavones were measured in the alkaline extract before pH adjustment, after pH adjustment, and after addition of PVPP. The isoflavones were analysed as described by Jankowiak et al. [2]. Dried samples were diluted in 50% methanol and analysed with a Waters Atlantic dC18 column (2.1 x 150 mm, 3 μ m) (Massachusetts, USA). The gradient was composed of the following two eluents: 0.1% formic acid in Milli-Q water and 0.1% formic acid in acetonitrile. The injection volume was 3 μ l and the flow rate was maintained at 0.3 ml/min. Standard solutions containing all 12 isoflavones in pure form were used for calibration and identification. The peak areas of the standard solutions and the extracts were measured using Dionex Chromeleon 7 Chromatography Data System, Thermo Scientific (Massachusetts, USA), at a wavelength of 254 nm.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) using IBM SPSS version 20 (SPSS Inc., Chicago, USA). Differences within the group were determined using Least Significant Difference (LSD) multiple comparison analysis. Differences at a value < 0.05 were considered significant.

RESULTS AND DISCUSSION

The impact of proteins in the extract

The alkaline liquid that is obtained after the removal of the fibrous fractions is a turbid suspension with a protein content of $31.4 \pm 3.6\%$, and sugar content of $38.5 \pm 3.7\%$ (both dry base). Therefore, after adjustment of the pH and addition of PVPP, the proteins precipitate to an extent that depends on the pH (**Fig. 1**). We investigated the adsorption at a pH as low as 4, because the adsorption of polyphenols onto PVPP is commonly better at a low pH due to protonation of the polyphenolic components [3-5]. However, not only protein-polyphenol interactions have been described in literature [11], but also the co-precipitation of isoflavones along with proteins during a protein isolate production process has been reported [14-18]. To quantify this effect, we measured the isoflavone concentration in the alkaline extract before adjustment of the pH and after decreasing the pH to 4, 4.5, 5, 5.5, 6, and 7, respectively. At pH 7, 3% isoflavones were lost, while at pH 4 the percentage lost increased up to 25% (**Fig. 1**). To describe the affinity of the isoflavones solely to the PVPP in the adsorption experiments, and to exclude the effect of co-precipitation with the proteins, we used the concentration of isoflavones in the liquid after protein precipitation as the isoflavone concentration c_0 in the model that is discussed in the following section.

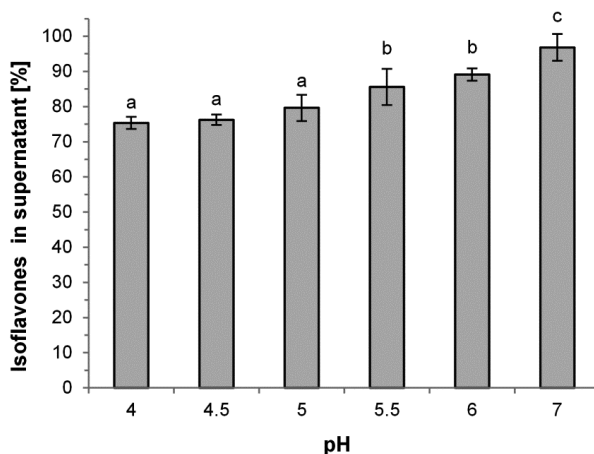


Fig. 1. Percentage of total isoflavones in the supernatant as function of the pH after acidification of the extract. Error bars represent standard deviations of 3 replicates. Bars with the same letter are not significantly different ($p > 0.05$).

The total isoflavone content considered in **Fig. 1** refers to 9 of the 12 isoflavones found in soy. Acetyl-daidzin and acetyl-glycitin were not found in the okara extracts, and acetyl-genistin was present in negligible amounts. The latter was monitored, but not taken into account when the total isoflavone content is expressed. **Fig. 2** shows the 9 main isoflavones present in the alkaline water extract, and their concentrations after precipitation of the protein at pH 5.5 and pH 4. The aglycones significantly decreased in the liquid ($p < 0.05$) upon lowering the pH and more solids (proteins and total solids) precipitated. Genistein showed the largest reduction. Its additional hydroxyl group distinguishes genistein from the other two molecules daidzein and glycitein. Therefore, it has often shown unique behaviour when it comes to solubility or interactions with other components [2, 18, 19]. The association of genistein during protein precipitation agrees with reports of Speroni et al. [14], though, in our case no significant interaction was seen with the malonyl-glucosides ($p > 0.05$).

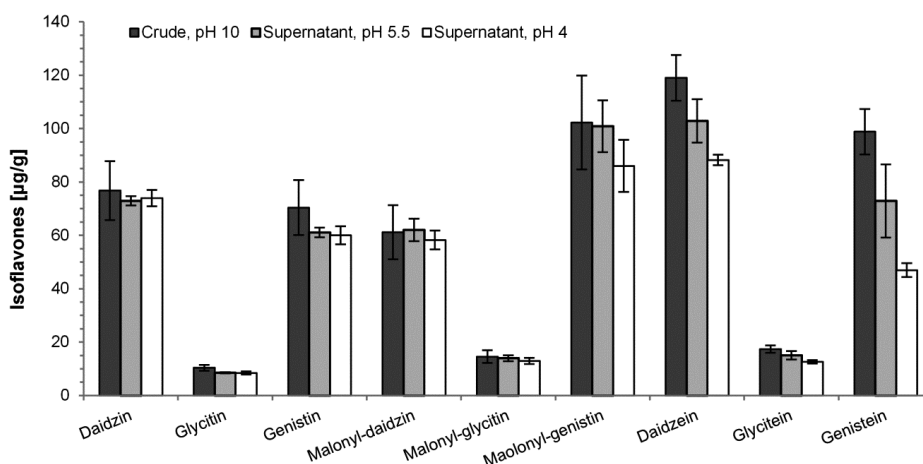


Fig. 2. Amount of isoflavones ($\mu\text{g/g}$) in the alkaline extract and in the supernatant after acidification to pH 4 and 5.5. Error bars represent standard deviations of at least 3 replicates.

The interaction of polyphenols with proteins depends on many parameters: the conformation and structure of the protein, the exact structure of the polyphenol and its amount and position of hydroxyl groups, the medium in which the association takes place, and the pH amongst others [8, 14, 16, 20]. In the reported cases of interactions of proteins with isoflavones, it cannot be excluded that the isoflavones that partition into the protein fraction solely co-precipitate due to their low solubility in water. The change in pH results in less charge of the isoflavones, which consequently themselves have a lower solubility in water as well.

In contrast, the aglycones neither showed increased solubility nor increased release from the okara matrix until a pH of 8 [2], which supports the hypothesis of interactions with the proteins as main cause for the reduction at lower pH, because an increased amount of protein that precipitates at pH 4 compared to pH 5.5 also resulted in an increased amount of aglycones lost in the supernatant. The aglycones are the most hydrophobic forms of the isoflavones, which makes them less soluble in water, but also increases the possibility of interaction with exposed hydrophobic parts of the proteins.

The proteins are in excess compared to the isoflavones. In case interaction exists, it will be rather weak. Nevertheless, the protein in okara is generally denatured. This becomes important considering that soy protein having a globular structure is less prone to bind polyphenols than proteins that have lost part of their conformation [20]. The isoflavone structure and soy protein specifically are not expected to interact strongly [20], but the denaturation state of the proteins may be an important factor in this case.

Slight discrepancies can be found in literature regarding the partitioning of isoflavones and proteins, especially in regards to the amount of isoflavones precipitating with the protein [14-18]. Those results are however not contradicting, particularly since subtle changes in a system's conditions can affect the intensity and type of interaction, probably the pH, glycinin and β -conglycinin ratios, and denaturation state being the most important ones to mention here. Furthermore, the dilution of the system in which proteins and isoflavones are dissolved in will largely influence their interaction. The more water surrounds the isoflavones, the weaker their association will be with the proteins [14, 16].

Affinities of total isoflavones, aglycones, glucosides, and malonyl-glucosides to PVPP

Fig. 3 shows the adsorption of the total isoflavones as a function of PVPP at a pH of 5. If we combine M_L g extract with initial concentration of isoflavones $c_{L,0}$, with M_{PVPP} g PVPP with original concentration zero, the isoflavones will partition over the two phases according to

$$M_L c_{L,0} = M_{PVPP} c_{PVPP} + M_L c_L \quad (1)$$

We assume a constant partitioning coefficient k , defined with

$$c_{PVPP} = k_{PVPP} c_L \quad (2)$$

A high partitioning coefficient implies a strong interaction with the PVPP; a low value means a low interaction, and less extraction from the fluid. Combining the two relations gives

$$M_L c_{L,0} = M_{PVPP} k_{PVPP} c_L + M_L c_L = (M_{PVPP} k_{PVPP} + M_L) c_L \quad (3)$$

$$\rightarrow \frac{M_L}{M_s} c_L = \left(\frac{1}{\frac{M_{PVPP}}{M_L} k_{PVPP} + 1} \right) \frac{M_L}{M_s} c_0 \quad (4)$$

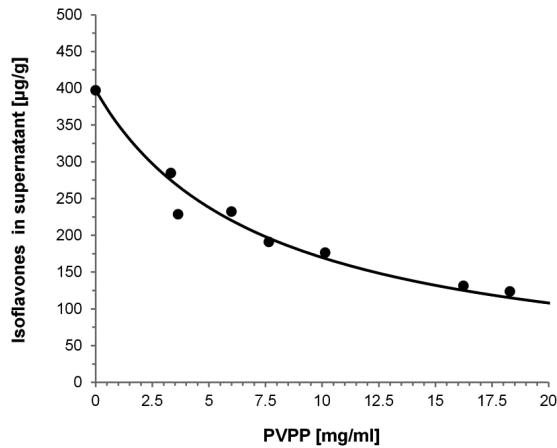


Fig. 3. Isoflavones remaining in the supernatant as a function of PVPP at pH 5.

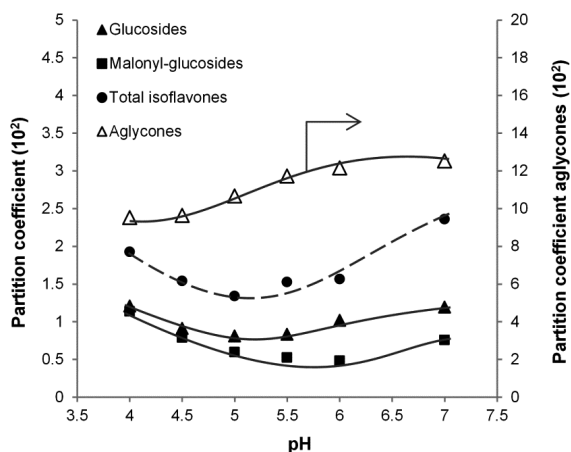


Fig. 4. Partition coefficient k for the glucosides, the malonyl-glucosides, the aglycones, and total isoflavones at pH 4, 4.5, 5, 5.5, 6, and 7, respectively. Lines are represented to guide the eye.

The adsorption of isoflavones onto PVPP was described with equation (4) for pH 4, 4.5, 5, 5.5, 6, and 7, respectively, resulting in partition coefficients shown in **Fig. 4**. (Exact values are shown in **Table A1** in the appendix). The overall affinity of the total isoflavones to PVPP represents the combined affinities of each isoflavone. However, the affinity is not similar for all isoflavones and especially the glycosylation of the aglycones results in a much lower affinity to PVPP as shown in **Fig. 4**, which is in agreement with previous studies [5]. The experimental data of the adsorption of each isoflavone group onto PVPP was well described by equation (4) (**Fig. A1** in the appendix) resulting in the partitioning coefficients summarised in **Fig. 4**. A clear dependence on the pH can be observed.

The affinity of the aglycones to the PVPP is about 10 times larger compared to the affinity of the glucosides and malonyl forms. This seems logical given that the aglycones have pK_a values above 9 [21] and are uncharged at lower pH, which increases their ability to form hydrogen bonds. Glucosides are also uncharged, but their attached glucose group makes them less hydrophobic than the aglycones, even at low pH. Moreover, the glucose groups represent a steric hindrance for stacking of the phenolic rings on the structure of PVPP [22]. The least affinity was observed for the malonyl glucosides, which are very water-soluble and charged at neutral pH. In contrast to the other forms, they have a low pK_a value [17, 23] and are still partly deprotonated around pH 5. In **Fig. 4**, a clear increase in their affinity towards PVPP can be seen with decreasing pH. The malonyl forms are only fully protonated at pH lower than 2

[23], which implies that the affinity would further increase with decreasing pH. The increase in affinity from pH 6 to pH 7 cannot be explained by the ionisation of the isoflavones; a different effect such as hydrophobic interactions may become more important. Since all isoflavones give a slightly increasing affinity at pH 7 relative to pH 6, this seems to be general for all forms, which might indicate an interaction with the structural backbone of the isoflavones, which has a large phenolic system which is susceptible to non-polar interactions. Furthermore, it is possible that the decreased affinity around pH 4.5 to 5.5 arises from a competing action of the proteins, which may also have the ability to interact with PVPP.

Adsorption of isoflavones to PVPP in the presence of okara

Commonly, pH adjustment improves the adsorption performance of flavonoids to PVPP due to protonation of the acidic functional group [3, 22]. **Fig. 4** and **Fig. A1** (appendix) show how the adsorption differs over the different isoflavone groups. For the malonyl-glucosides a lower pH leads to better adsorption. However, a pH change from alkaline to acidic conditions results in salt waste streams, and salt residues in the product. In addition, the overall adsorption is even better at pH 7 than it is at lower pH (**Fig. 4**).

In other words, the pH steps do not have that much influence on the isoflavone partitioning, but will mainly affect the protein precipitation. Therefore, we investigated the adsorption of the isoflavones to PVPP with the entire fibrous okara matrix present. We hypothesised that the adsorption of the isoflavones on the PVPP, and the resulting lower concentration in the extract may offer driving force for additional isoflavones to be extracted from the okara, without also extracting more proteins. This will lead to a higher yield and a better purity.

The results are shown in **Fig. 5**. The curves for the malonyl-glucosides and glucosides are comparable with the according adsorption curves at pH 7 (**Fig. A1f**), where the fibres had been removed before adsorption. The malonyl-glucosides are expected to fully solubilise in water, as well as the major part of the glucosides [2]. However, the aglycones showed an unusual curve (**Fig. 5**). It should be kept in mind that the pH in the system with fibres is different (floating pH) from the pH in the adsorption experiments shown in **Fig. A1**. The first extraction step, which is performed under alkaline conditions in one case to solubilise the more hydrophobic aglycones (**Fig. A1**), is omitted in the other case (**Fig. 5**). The impact of the pH on the extraction of isoflavones with water can also be found in our previous work [2]. At a 20:1 water-to-solid ratio, less than half of the aglycones are in the liquid compared with an ethanol extraction or alkaline water extraction. Therefore, the concentration of aglycones

before PVPP addition (c_L with $M_{PVPP}=0$) in **Fig. 5** represents the concentration in the liquid, but not the total amount in the whole system as more aglycones are still bound to the matrix. That explains why the starting concentration of aglycones before PVPP addition (c_L with $M_{PVPP}=0$) in **Fig. A1** is higher.

In our previous work [2] we have described the affinity of the isoflavone groups to the matrix, resulting in a partition coefficient k_s of 197 describing the affinity of the aglycones towards the matrix (solids). With this model, we also estimated the actual amount of aglycones that should be in the matrix (c_0), which in the previous paper was 478 $\mu\text{g/g}$. In the present paper, c_0 was estimated to be 781 $\mu\text{g/g}$. An ethanol extraction confirmed a higher amount of aglycones in this okara batch than in the one used for the previous experiments. A new mass balance (equation 5) can now describe the situation shown in **Fig. 5**.

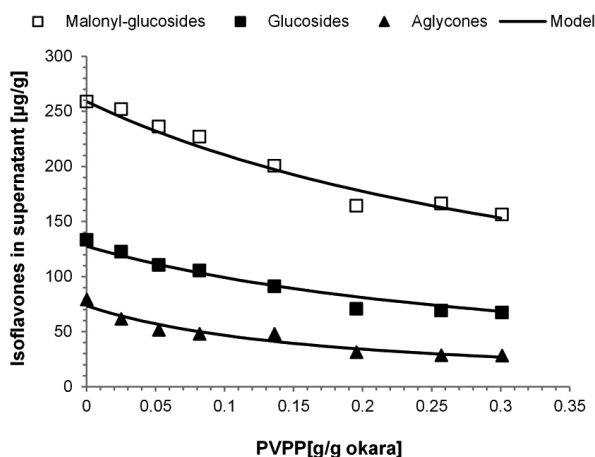


Fig. 5. Malonyl-glucosides, glucosides, and aglycones remaining in the supernatant as a function of PVPP with the okara matrix present.

If we have M_s g of okara, having initial isoflavone concentration c_0 , and contact it with M_L g water and M_{PVPP} g PVPP, the isoflavones will now distribute over three phases, according to

$$M_s c_0 = M_{PVPP} c_{PVPP} + M_s c_s + M_L c_L \quad (5)$$

If we now use two (constant) partition coefficients,

$$c_L k_{PVPP} = c_{PVPP} \text{ and } c_L k_s = c_s \quad (6)$$

We will end up with the following relation:

$$M_s c_0 = (M_{PVPP} k_{PVPP} + M_s k_s + M_L) c_L \quad (7)$$

or

$$c_L = \frac{c_0}{\frac{M_{PVPP}}{M_s} k_{PVPP} + k_s + \frac{M_L}{M_s}} \quad (8)$$

in which M_{PVPP}/M_s represents the mass ratio between PVPP and okara, and M_L/M_s the mass ratio between liquid and okara.

We assume that the partition coefficients between solution and PVPP do not change with the presence of the okara, and indeed, with the use of the affinity of the aglycones to PVPP at pH 7 ($k_{PVPP} = 1252$) (**Table A1**), and the affinity to the matrix that was determined in previous work ($k_s = 197$; [2]), the experimental data can be described well with equation 8, suggesting a simultaneous adsorption and release of aglycones from the matrix. Due to a concentration drop of the aglycones in the solution after adsorption, aglycones are able to solubilise further from the matrix leading to a higher concentration in the remaining mixture and an apparent lower adsorption. Next, the amount of aglycones adsorbed to PVPP, and the residue in the matrix as function of the mass of PVPP can be described with the partition coefficients. The curve showing the concentration in the liquid is flattened due to the concurrent extraction from the matrix and adsorption onto the PVPP (**Fig. 6**). Besides, it can be observed that PVPP is able to bind a significantly larger amount of isoflavones present in the total system, compared to when the isoflavones are first extracted from the okara, and the solution is then separately contacted with the PVPP.

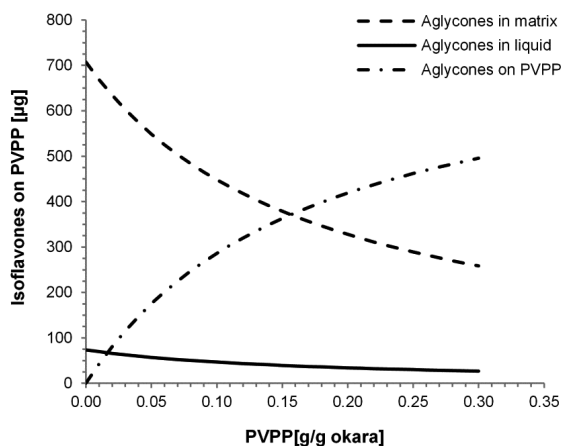


Fig. 6. Prediction for the amount of aglycones on PVPP, in the matrix, and in the liquid using k_{PVPP} and k_s .

For the malonyl-glucosides the simultaneous extraction was not found, because even at a low liquid-to-solid ratio (20:1) they are all solubilised in the liquid phase. Still, the immediate or simultaneous adsorption of the glycosides without prior removal of the fibres has an advantage: the removal of the fibres involves the loss of isoflavones that are removed along with the fibres leading to a reduction of the process yield. By addition of the adsorbent to the entire system, those remaining isoflavones may be adsorbed as well, given a large enough capacity of the adsorbent.

Regarding the β -glucosides, the adsorption curve with the matrix present (**Fig. 5**) is only flattened to a very small extent compared to the curve without the fibres present (**Fig. A1 f**). The case of the β -glucosides is in between the malonyl-glucosides and aglycones. The β -glucosides are much better soluble in water than the aglycones, but they do have a solubility limit at low water-to-solid ratios (< 50:1). This is in contrast to the malonyl-glucosides, which easily solubilise even at low ratios. The principle of simultaneous adsorption becomes now relevant to support the reduced use of water. A simulation of the β -glucoside concentration at a liquid-to-solid ratio of 10:1 is shown in **Fig. 7**. The intermediate affinity of the β -glucosides towards the water makes them remain in the matrix at low ratios and the starting concentration in the liquid is lower (**Fig. 7**). Again, the adsorption of the β -glucosides onto PVPP and the depletion of the matrix are shown by using the affinity to PVPP at pH 7 ($k_{PVPP} = 119$) and the affinity to the matrix that was determined in previous work ($k_s = 21$; [2]), respectively. However, due to a 10 times lower affinity of the glycosides to the PVPP the effect is not so pronounced.

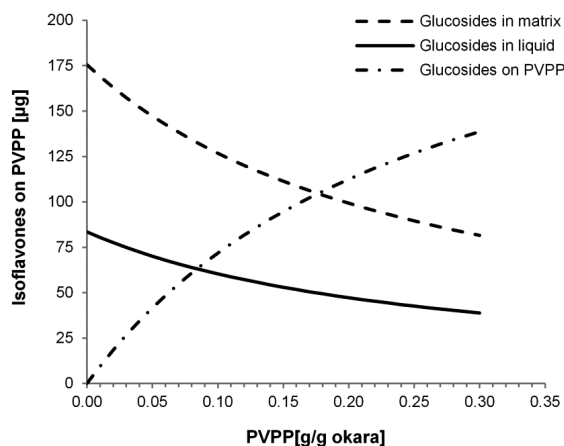


Fig. 7. Prediction for the amount of β -glucosides on PVPP, in the matrix, and in the liquid using k_{PVPP} and k_s .

Consequences for process design

Separation processes to isolate high value components are often derived from analytical principles. On analytical scales, extra process steps are not a big complication. However, on industrial scales, simpler processes lead to more economic and sustainable processes [24, 25].

Therefore, we investigated the influence of the okara matrix on the adsorption process, and combined extraction with adsorption, which leads to an integrated process that is simpler, yields more isoflavones, and requires only one process step (not counting the physical separation of the PVPP phase from the okara and fluid). The partition coefficients that were found, also suggest a better extraction process for the components in the mixture that are rather hydrophobic and therefore are less soluble in water than the other components, making a commonly used additional extraction step with alcoholic solvents superfluous.

This combination of extraction and adsorption only works by balancing solvent strength and hydrophobicity of the adsorbent. Isoflavones are commonly extracted with alcoholic aqueous solutions [26], but adsorption of the isoflavones from an alcoholic aqueous solution to PVPP would be much weaker. In fact, ethanol can now be used to *desorb* the flavonoids from the PVPP [3, 22, 27]. Hence, in this intensified process, water is a better all-round choice as solvent.

Further exploration of this principle is suggested. Proteins have shown to also act as a carrier under specific conditions, instead of PVPP, suggesting that further exploration of the matrix could be used in an advantageous way avoiding several expensive and elaborate steps for purification of components. Proteins interact similarly as the PVPP with the isoflavones (i.e. by phenolic ring stacking and hydrogen bonding). Therefore, it is possible that the isoflavones precipitated with the proteins at low pH are then not lost, but that complete elution can be achieved, from the PVPP as well as from the proteins, and that the protein reduces the amount of resin needed. While adsorption processes are conventionally done with packed beds, this is not possible here, since the fluid is a suspension of okara in an aqueous solution. Therefore, the resin particles need to be suspended in the mixture as well.

In this study, a regeneration-grade PVPP was used. Compared to the micronised PVPP with a particle size of 25µm to increase the surface area for enhanced adsorption, the PVPP used in this study has a larger particle size of 110 µm. The larger particle size and mechanical strength makes it able to withstand repeated recycling rendering it a low cost material [22]. The isoflavones desorb easily from the PVPP with ethanol (data not shown), and other studies have reported a good balance between adsorption and elution performance of PVPP [6, 22, 28]. However, separation of the particles from the okara matrix will yet be difficult at this size, and it is suggested to use a resin with similar good adsorption/desorption performance, but larger particle size to be easier separated from such a fibrous matrix. One may hypothesize that particles with a heavy core, might enable us to use sedimentation by gravity as separation mechanism.

To summarise, lowering the pH, which results in some loss of isoflavones due to co-precipitation with proteins, was found to be unnecessary, since the isoflavones interact sufficiently with the PVPP at neutral pH. Addition of the PVPP to the okara and water creates an additional driving force leading to higher yields, and avoiding co-precipitation with proteins. Adsorption was effective for both glycosides and aglycones. A distinct behaviour of the aglycones led to the assumption that further extraction of the aglycones in the matrix took place simultaneously to adsorption.

CONCLUSIONS

The use of polyvinyl polypyrrolidone (PVPP) for the isolation of isoflavones from okara was investigated.

First, aqueous extracts were made from okara, and the resulting partition coefficients were determined as function of the pH, for the three groups of isoflavones (malonyl-glucosides, glucosides, and aglycones). While a lower pH led to some extent to better adsorption, the overall amount of isoflavones isolated was somewhat lower due to co-precipitation of proteins. A rise in pH from 6 to 7 led to somewhat better partitioning towards the PVPP for all isoflavones, which is probably due to interaction with the phenolic backbone of the isoflavones with the PVPP. The more hydrophilic and water-soluble glycosides had less affinity to the PVPP, but adsorption was hardly influenced by the present matrix. The more hydrophobic (and less water-soluble) aglycones had very high affinity to the PVPP.

Simultaneous exposure of the PVPP to the okara and an aqueous phase led to better extraction, and higher yields, especially for the aglycones, which are not well solubilised by an aqueous phase. The presence of PVPP continuously removes the isoflavones in the solution, therefore replenishing the driving force of the isoflavones to desorb from the okara matrix, and ultimately leading to a higher yield. This concurrent, one-step extraction-adsorption only works with a solvent that does not have a strong interaction with the target components: use of mixtures of ethanol and water, as are commonly used for isolation of isoflavones, would lead to reduced adsorption of the isoflavones to the PVPP, and may lead to reduced overall recovery.

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Appendix A

Table A1. Partition coefficient k for the total isoflavones and isoflavone groups describing the affinity to PVPP at pH 4, 4.5, 5, 5.5, 6 and 7, respectively.

Partitioning coefficient	pH 4	pH 4.5	pH 5	pH 5.5	pH 6	pH 7
<i>Total isoflavones</i>						
$k (10^2)$	1.9 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.5 ± 0.2	1.6 ± 0.1	2.4 ± 0.3
<i>Malonyl-glucosides</i>						
$k (10^2)$	1.1 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.8 ± 0.1
<i>Glucosides</i>						
$k (10^2)$	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
<i>Aglycones</i>						
$k (10^2)$	9.5 ± 0.3	9.6 ± 0.5	10.7 ± 1.3	11.7 ± 1.3	12.2 ± 0.6	12.5 ± 0.9

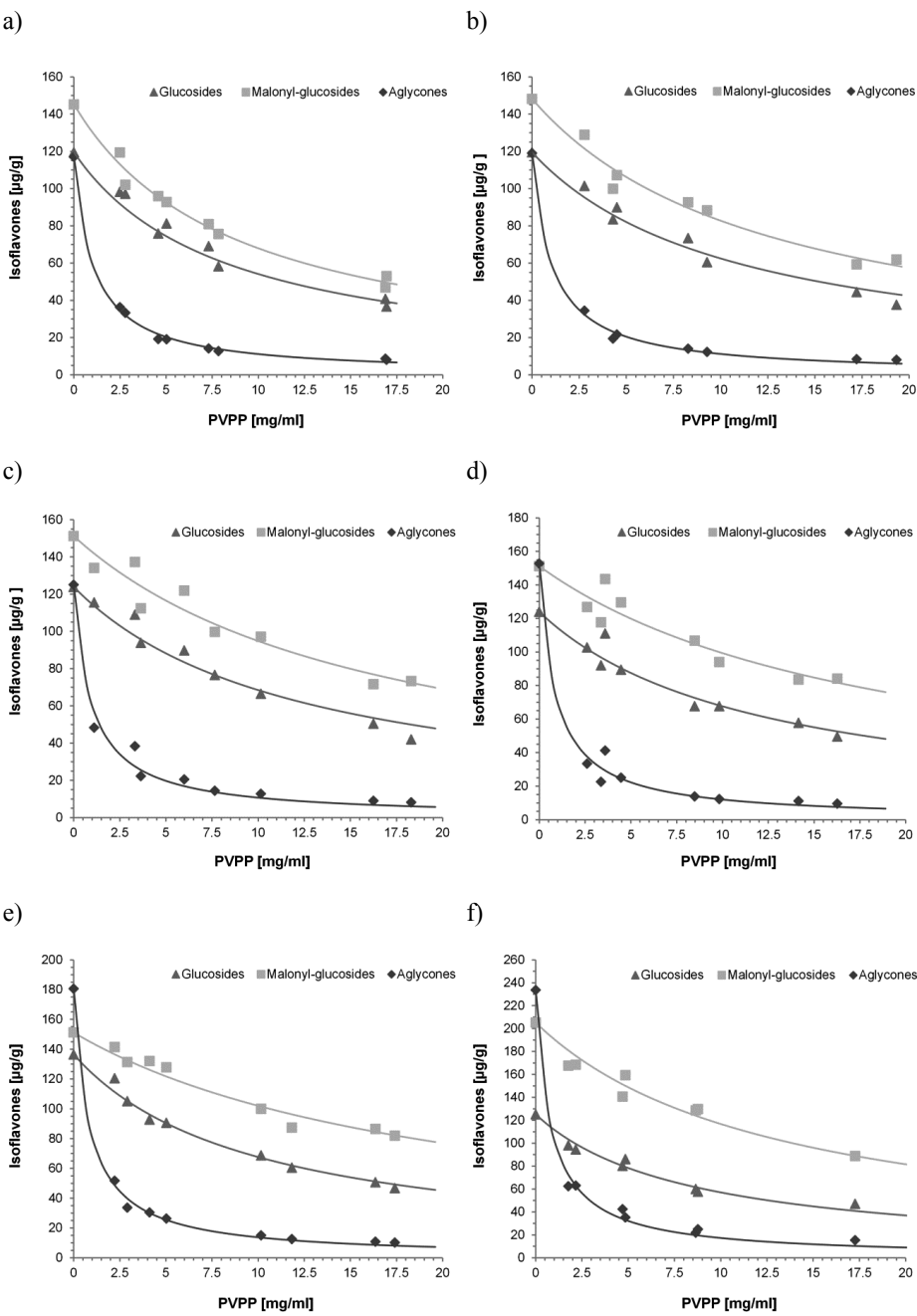


Fig. A1. Malonyl-glucosides, glucosides, and aglycones remaining in the supernatant as a function of PVPP added at a) pH 4, b) 4.5, c) 5, d) 5.5, e) 6, and f) 7, respectively.

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Chapter 6

A process synthesis approach for isolation of isoflavones from okara

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Abstract

Due to the complexity of food matrices, process synthesis methodologies have not been as widely applied in the food industry as in the chemical industry. Here, we describe the application of a process synthesis methodology to design a system to separate valuable components from a by-product of the soymilk production.

The method yielded a number of potential processing pathways and relevant mechanistic questions, which required experimental input. The combination of considering the overall system on the level of general transformations, heuristics, and additional insights through experiments resulted in a simplified conceptual process design for the separation of isoflavones from okara with a globally more sustainable choice. The holistic approach within process design as an implication of the methodology is discussed.

INTRODUCTION

Food processing has to become more sustainable and energy-efficient, use non-toxic solvent routes, and make efficient use of raw materials, leading to more natural ingredients and products. On the way towards no-waste processes, the isolation of valuable products from side streams has gained a lot of interest. It is important to consider several factors in this context. While the isolation of valuable components from a relatively low value stream appears attractive, the requirement for sophisticated extraction and purification technologies may reduce the profit margin and lead to an uneconomic outcome [1]. Additionally, the extra process steps for the isolation of the valuable components should be according to modern objectives on good resource use and energy efficiency, and the use of natural, less toxic ingredients and processing aids. To comply with the combination of these constraints, a systematic approach towards such processes is necessary. Here we demonstrate the potential of process synthesis for the isolation of valuable components from a side stream of soy processing, namely okara. Okara can be considered as a model material for other by-product streams such as brewer's spent grain or pomace, in which minor components have economical potential as well.

Process synthesis methodologies were developed mainly for chemical products, until the similarities between chemical consumer goods and structured food products were recognised [2-6]. The latter explains the growing interest of the food industry to translate conceptual design methodologies from the chemical sector into the food sector. However, the process synthesis methodologies have to be updated concomitantly due to the dynamics within the industry, such as a shifting product portfolio, new economic situations, new regulations, and sustainability concerns [7-9]. The main challenges for the food industry are described in Meeuse [10] and Bongers & Almeida-Rivera [11]. The complex matrix of a typical food material plus the manifold interactions between food components complicates the systematic synthesis of processes, amongst other due to the difficulty of quantitatively capturing those aspects in mathematical models. Consequently, a product-driven process synthesis methodology was developed for the design of structured products, which was successfully applied by Gupta & Bongers [12] for the redesign of a Bouillon cube production process. However, until this moment, this approach has not yet been applied for the design of processes aimed at utilisation of side streams.

In this paper, we extend the concept of the product-driven process synthesis methodology to the isolation of valuable components from a side stream, which in essence means the design of a separation and isolation process. In previous examples on product assembly, the

challenges were to influence the interaction between the ingredients while processing and to predict the resulting texture and other properties of the food product according to the consumer's liking. The challenges faced in the development of a separation process are discussed by Jankowiak et al. [13]. There, it was demonstrated that the effect of an extra processing step on the outlet streams is also difficult to predict, again due to the behaviour of interacting components and the thermodynamic and kinetic behaviour of the system. A further challenge arises from the fact that additional components, such as extraction solvents, have to be introduced to perform a certain separation that often introduce new impurities and may ask for additional processing steps.

For this study, we used okara as a model material. Okara is a by-product of soymilk production, which contains mainly fibres, proteins, and fat [13, 14]. Amongst the minor components, okara contains isoflavones [15]. They belong to a group of polyphenols, which reportedly play an important role in human health [16], and which therefore have considerable value.

THEORY

Process synthesis methodology

To facilitate the process development, we adapted a process synthesis methodology based on Bongers & Almeida-Rivera [11]. These authors suggested a new methodology combining product and process synthesis based on a process systems engineering strategy. Their methodology is based on an approach of Douglas [17], which simplifies the complexity of the problem by dividing the problem into several hierarchically ordered levels. Starting with very generic definitions and network structures, more detailed and local decisions are required at each level, which allows the user to go strategically from one level to the next one. As knowledge about the system increases, the designer can fill in more detail at each level, and with this go from generic transformations and a very rough network of physical or chemical transformations all the way towards a complete feasible flow sheet alternative. An important component of this methodology is aimed at the generation and screening of alternative flow sheets. Conceptualisation retards the fixation of a flow sheet, which reduces the risks of irreversible and suboptimal decisions during the design of the process.

The methodology uses a step-wise design method over several levels, which are summarised in Table 1 and discussed in more detail in Bongers & Almeida-Rivera [11]. The first three levels (0-2) deal mainly with marketing questions, the business context, the project background and the needs of the customer, which are translated into product properties. In our case of the isolation of valuable minor components, the focus is at the levels of ‘Input-Output’, after which ‘Task networks’, and ‘Mechanisms’ can be further derived. When the input and output level is defined, fundamental task networks are listed and evaluated based on heuristics, literature and experimental work. In the following level, mechanisms are selected that can fulfil the defined tasks. At this stage, it should be pointed out that ‘tasks’ and mechanisms should not be mistaken for unit operations, which, if selected, would lead the designer in an already defined direction. The repeated revision of levels defines the process in more depth, leaving the equipment selection and design to the final step. The methodology combines conventional product design and process design into product-oriented process synthesis as it has been the trend within the last two decades [7, 18-20].

Table 1. Levels of the PDPS methodology

Level	Description
0	Framing
1	Consumer wants
2	Product function
3	Input-output
4	Task network
5	Mechanisms and operational window
6	Multiproduct integration
7	Equipment selection or design
8	Multi product-equipment integration

The case study okara

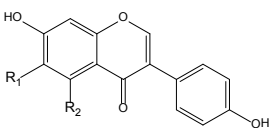
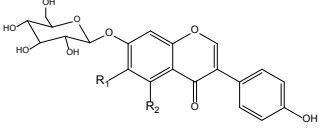
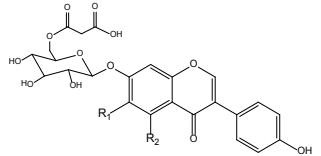
Okara, as stated a by-product from the soymilk production, contains about 80% of moisture. The dry matter of okara consists of insoluble fibre (with high water binding capacity) and other insoluble and soluble components such as proteins and sugars. Currently, okara is used as animal feed or discarded as landfill, which will become unacceptable from an economic and environmental point of view due to an increased scarcity of resources, and more and more stringent environmental regulations. Consequently, okara has to be upgraded for use as food or non-food applications, or to be separated into pure or enriched fractions of valuable components. The main valuable components in okara are isoflavones, which belong to a large group of polyphenols. Twelve main chemical forms of the isoflavones are identified in soy and okara, classified in four groups, aglycones, glucosides, acetyl-glucosides, and malonyl-glucosides [21]. In okara, the main groups present are the aglycones, glucosides, and malonyl-glucosides, and are thus the focus of this study [15]. The glycosidic forms are the dominant isoflavone form in the raw material, the soybeans, and can be transformed into the other forms under certain processing conditions [22, 23]. The isoflavone's structure and an estimation about their hydrophobicity by the logD value [24] are shown in **Table 2**.

Extraction of the isoflavones requires a novel separation process with a minimal environmental impact. Unfortunately, the design of this process is hindered by the lack of clarity about the embedding of the isoflavones in the okara matrix. Theoretically, the isoflavones can be bound in three different manners:

1. Isoflavones are dissolved in the water that is bound by the matrix;
2. Isoflavones form (soluble) complexes with the soluble proteins;
3. Isoflavones are bound to the (insoluble) biopolymer matrix.

It is even possible that isoflavones are present in all three forms, because isoflavones consists of a mixture of components with different properties (e.g. hydrophobicity).

Table 2 Main isoflavones present in okara, their structure and distribution coefficient (logD) at pH 7

Group	Isoflavone form	R1	R2	Chemical structure	logD (pH 7) ^a
Aglycones	Daidzein	H	H		≈ 2.02
	Glycitein	COH ₃	H		≈ 2.33
	Genistein	H	OH		≈ 2.67
β-glucosides	Daidzin	H	H		≈ 0.45
	Glycitin	COH ₃	H		≈ 0.29
	Genistin	H	OH		≈ 0.80
Malonyl-glucosides	Malonyl-daidzin	H	H		≈ -2.25
	Malonyl-glycitin	COH ₃	H		≈ -2.40
	Malonyl-genistin	H	OH		≈ -1.60

^a data obtained from chemicalize.org [24]

In a traditional manner, one would suggest the following route for separation: First, a solvent will be selected to further solubilise the isoflavones, but the high moisture content of okara limits the freedom in solvent selection, or leads to the requirement of high liquid-to-solid ratios. To increase the freedom for solvent selection, okara can be dried, after which the solvent extraction can be performed using the optimal solvent. Then the solid components will be removed through filtration or centrifugation, leading to a clear solution with soluble components only. Finally, the components will be further purified, most likely with an affinity-based process (e.g. with adsorbents or liquid-liquid extraction). A consequence of this design is the use of large solvent quantities leading to drying and recycling of large liquid streams. Most likely, alternative processes can be designed that are more efficient in energy and solvent use. In this paper, we describe how a process synthesis methodology can be used to derive other alternatives and how this methodology interacts with the input from experiments.

MATERIALS AND METHODS

For most of the experimental and theoretical work accompanying the process synthesis described in this paper, we will refer to our previous studies [13, 15, 25-27]. With the experimental work described in this section, we tested the simplified approach for a process with combined tasks.

Okara production

For experimental data, okara was produced with an ASC50 soymilk system (ProSoya Inc., Ottawa, Canada) as described by Jankowiak et al. [15].

One-step separation

For lab-scale experiments, 160 g crude okara with a moisture content of 78% was mixed with water in a ratio of 20 g water to 1 g dry matter (okara). The mixture was stirred at 500 rpm for 1h at room temperature. Subsequently, 3.7 g of amberlite XAD7HP resin (Sigma-Aldrich Co., Schnelldorf, Germany) was added on top of the plunger (see **Fig. 1**), which had a mesh small enough to keep okara and resin separated for later elution of the resin. The resin was then immersed in the liquid and the mixture left stirring at 900 rpm overnight for adsorption. After the resin was removed, it was washed with Mili-Q water, and the wash water kept for analysis.

The washed resin was immersed into 96% ethanol (50% of the amount of water used for extraction), and the liquid was stirred for 3h at 50 rpm. Samples for the determination of the solids content and for the isoflavone analysis were taken at each step during the entire process.

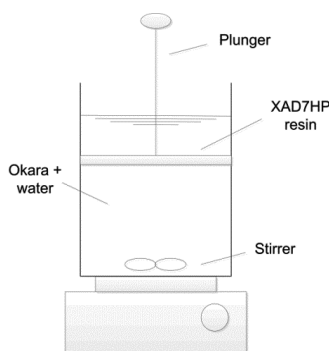


Fig. 1. Experimental set-up for integrated extraction-adsorption

Isoflavone analysis

Samples for isoflavone and solids determination were treated and analysed as described by Jankowiak et al. [27]. Acidified Milli-Q water and acetonitrile were used as eluents to separate the isoflavones on a Waters Atlantis dC18 column (3 μ m, 2.1 x 150 mm). The detection wavelength was 254nm.

RESULTS AND DISCUSSION

Many current processes comprise several steps and require more than one solvent. To contribute to the general aim of a more sustainable food production, the use of chemicals and solvents, energy, and the production of waste has to be limited or avoided. Optimised and simplified processes can be one way to achieve such goal. In this section, the process design and resulting process options based on the mentioned process synthesis methodology are described starting at the Input-Output level.

Input-Output

Evaporation and distillation are inefficient processes regarding energy use, as also shown in this case study [25]. Previous research on the suitability of different solvents for the extraction of isoflavones from okara led to the insight that

- water as solvent can extract isoflavones reasonably well, with preference for the glycosidic forms of the isoflavones, which are naturally present in non-modified soybeans [28]. However, water also leads to swelling of the okara matrix and can disperse or even solubilise other biopolymers with the isoflavones, such as the fibres and proteins, respectively.
- Ethanol is an especially good solvent for the aglycone form (a form, which is a result of a hydrolysis reaction during extraction of the soybeans with water) and prevents swelling of the matrix. However, the high viscosity and a possibly even a glassy state of the okara matrix at low water content may inhibit the release of the isoflavones.

A mixture of both solvents may solubilise all isoflavones, prevent extreme swelling of the matrix, and minimise the solubilisation of other components such as sugars and peptides. We limited ourselves to ethanol and water as being the most non-toxic and food-grade polar solvents. However, using ethanol will imply the need for a process to remove the ethanol

from the raffinate, for example by evaporation/distillation, may complicate the maintenance of safety around the process, and will induce an extra cost for using this solvent.

These disadvantages, and the theoretical and experimental indications that most of the isoflavones are at least somewhat water-soluble, led to further investigation of okara and its isoflavones in a pure water phase [27]. The experimental input will be further discussed in the following section ‘task networks and mechanisms’.

The input of the process is defined in more detail along the entire development of the process. The input stream (raw material) is obviously the by-product okara. While this material in fact is highly structured, and contains many components, we will here limit its definition to the major components of okara (**Fig. 2**).

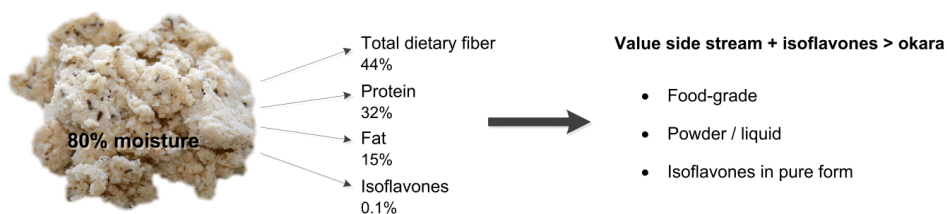


Fig. 2. Input-Output definition in the case of okara utilisation through separation of isoflavones

The quality and composition of the output depends on the type of solvent and the extraction procedure. It is often suggested that a higher purity may result in a higher economic value. However, this does not necessarily need to be the case. In a more conventional approach, the only optimisation route aims at increasing yield and in case of separation processes high purity. It is questionable whether high purity combined with high yield should be and can be the primary process aim in future processes, especially if resulting by-products have to be reduced or further upgraded. In case of okara, this means that the residue has to be upgraded in a different manner. New objectives such as the sustainability of a process and products can lead to trade-offs in other objectives. To conclude, the information above suggests that three aspects of the optimal point of process design exist: the purity of the product (fraction), the yield from the raw material, and the sustainability or environmental impact of the isolation process.

- The *yield* from the raw material is mostly important when the isolate has a very high value, and when it is important that the raw material has no residue afterwards. In this case, the okara is a stream that does not have much value. All isoflavones that are recovered have value. Therefore, this value is proportional to the yield: to go from 90% recovery to 95%, only gives 5% increase in income. From a different perspective, to go from 90% to 95% recovery, the residual content in the feedstock has decreased by a factor two, implying a much more severe isolation process and subsequently much higher cost of isolation.
- The product quality is closely coupled to the *purity* in this case. A product containing a lower purity and still contains protein and carbohydrates will have a lower value than an isolate with higher purity. However, for many applications (e.g. as food ingredient) very high purity is often not needed.
- The *sustainability* of the process is relevant after the product is ensured to have the right specifications (good purity), and the process economics is reasonable. Therefore, the sustainability of the process is a target that is optimised when both the product purity and the yield are defined.

It is important to mention that optimising those aspects has to be performed in an iterative way. Within each level of the methodology, several options are tested, reported, and decisions made. A final decision allows going on to the next level. However, the method also implies to go back to an earlier level to refine or re-define decisions made earlier. In fact, the levels have to be dealt with also in an alternating way to achieve an optimal global solution. Previous work showed that water can be a feasible solvent for extraction [27]. It yields slightly less isoflavones, but leads to a more efficient extraction regarding sustainability of the process and a much more simplified process design. Furthermore, the desired purity of the isolate depends on the application of the isolate.

In a traditional approach, solvents, chemicals, and several processing steps may lead to highly pure ingredients, but the residue would have lower value because of the harsh conditions needed for complete separation. Those harsh conditions may degrade other components, and may lead to residual solvents in the remaining material, which hinders further application as animal feed. If in this case we assume that the okara was used as cattle feed, the spent okara should be at least suitable as cattle feed as well after the isolation of the isoflavones. This can be achieved by the use of water as a solvent, thus generating maximum value when considering the creation of the isoflavone isolate without the loss of value of the okara. It is

probable that further refining of the okara, e.g. by separating the proteins and some of the carbohydrates, would generate even more value. However, if the spent okara can only be discarded as waste after isoflavone extraction, it will be difficult to compensate for this only by using the economic value of the isoflavone isolate. In general, it is essential that all fractions must be utilised to generate value when upgrading side streams. Isolating a valuable fraction while destroying the value of the rest will not result in a feasible processing system. In detail, this means that either all the components have to be separated and further utilised in food products, or the residue after separation of isoflavones has to be suitable for animal feed.

Task networks and mechanisms

After defining the input-output level, task networks were built based on several hypotheses (see example in section ‘the case study okara’). Some selected task networks are shown in **Fig. 3**.

The first and simplest task network (**Fig. 3a**) had to be defined further in more detail. Therefore, we developed sequences of possible tasks. At this stage, the most promising task networks are selected, along with a list of mechanisms needed to fulfil this task (an example is given in **Fig. 3c**). This is not a sequential procedure, but the process of building task networks and the evaluation of separation mechanisms is iterative, if necessary with additional input from experimental work. Furthermore, the experimental exploration is also important for finding new and unexpected, and more sustainable processes.

Following the classical route, different ethanol-water fractions ranging from 0% - 90% (w/w) ethanol were tested as solvents for the extraction of isoflavones from crude and from dry okara. [15]. An intermediate ethanol concentration (between 50% and 80% ethanol) gave the best extraction yield and purity of isoflavones in the extract. Nevertheless, the purity (isoflavones per dry weight extract) still did not exceed 1.4%, and further purification is required for a more concentrated isoflavone product that can be used for example as health ingredient in food products. The evaluation of the different solvents for extraction gave information on the matrix, which means its swelling behaviour, the affinities of the isoflavones towards the matrix, and the ability of other components to dissolve. The solubility of some of the isoflavone forms is lower in water than in ethanol or ethanol-water mixtures, which is a consequence of the finding that some types of okara have unnaturally high contents of aglycones, which is a consequence of the soymilk process.

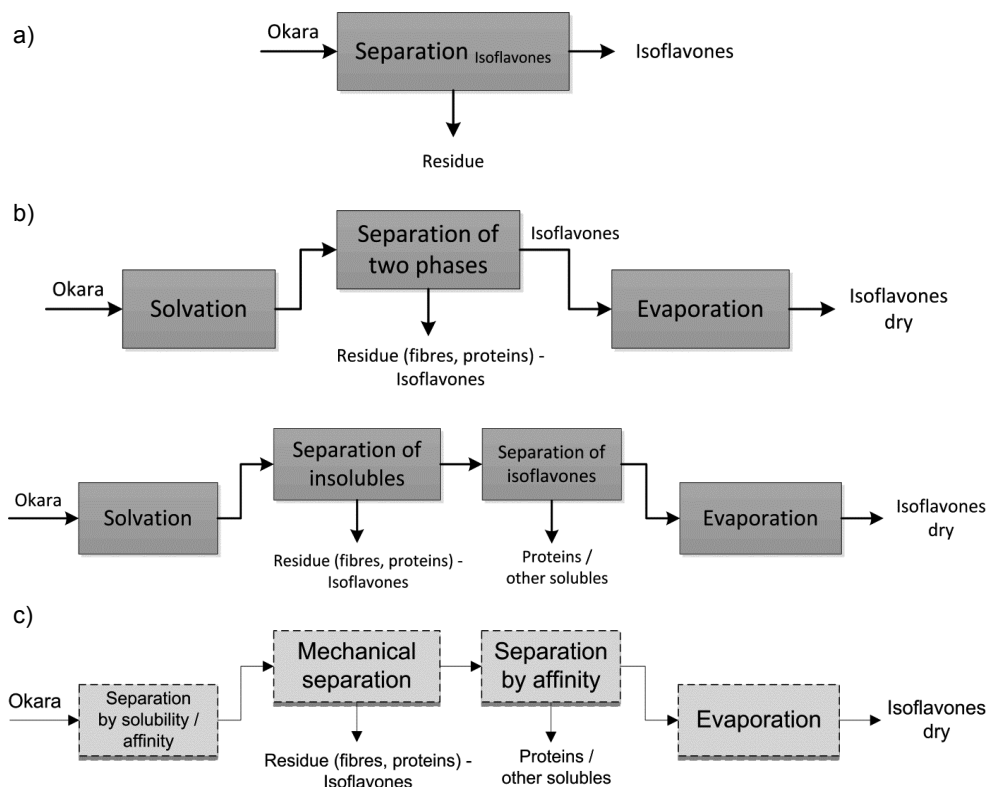


Fig. 3. a) Simple task network, b) more defined task networks, c) mechanisms

The presence of those components explains why ethanol or aqueous ethanol is often considered as preferred solvent [29]. However, a further analysis showed that one has to differentiate between the aglycones and glycosides, since their different natural structures, and therefore polarity and hydrophobicity, lead to their different extractive behaviour. The aglycones have a much lower solubility in water than in ethanol [15, 30], and co-solubilisation of other components make aqueous ethanol the preferred solvent. However, after the extraction of the isoflavones from okara, they have to be isolated from the extract. A suitable mechanism to separate phenolic compounds such as isoflavones from a solvent

mixture is using specific affinity (e.g. based on hydrophobicity). Therefore, the affinity of the different isoflavone groups to chromatographic resins depending on the solution they are dissolved in, was evaluated [26]. Isoflavone extracts produced with ethanol/water mixtures ranging from 0% - 80% ethanol were tested for their adsorption on different resins, and our assumption was confirmed that affinity towards a resin was largest when the components are dissolved in water. This leads to the conclusion that, if including the isolation (adsorption) step, it is not optimal to use an ethanol containing extractant. Water, being a relatively poor extractant, makes the subsequent isolation much easier. The holistic approach of including both steps points to a different optimal process design.

To summarise, when keeping further purification of the extract in mind, it could be concluded that each step has its own optimum conditions, which are not the best conditions for the overall process. For the extraction, an ethanol concentration of around 50% was optimal, but for isolation by adsorption, the water extract gives in the most efficient process.

A trend in process design is towards the integration of process steps as it leads to simplification and often more efficient and economic processes [8, 31]. It is well known that solvents are the most detrimental part to the efficiency of processes, since they tend to leave residues, and parts of them are often emitted to the environment. An isolation step, for example by chromatographic methods, is indispensable to reach highly purified isoflavones. Therefore, instead of optimising both processes themselves, we evaluated the entire system and concluded that a water-based extraction could be integrated with the subsequent chromatographic purification. Solvents are an important factor determining the efficiency of a process, and evaporation of those solvents has to be reduced as much as possible. Therefore, unit operations such as extraction and adsorption should be integrated despite the fact that the optimal solvent for okara had not been equal to the optimal solvent for adsorption.

The sustainability analysis of the extraction process [25], the natural water-solubility of part of the present isoflavone forms, but mainly the requirement of integrating the subsequent processing step of purification in the overall design, lead to the conclusion that water should be used for extraction. Nevertheless, the water extract as described above is not suitable for a packed bed chromatographic process: the stream is a suspension of aggregates, mainly of proteins. Therefore, another way of combining extraction and adsorption was evaluated. The same task should be performed, but heuristics can be used to use more suitable mechanisms to do so. For example, we precipitated the proteins that are present in a water extract, but not in the ethanol extract, by lowering the pH to 4.5. As a result, the clarified extract is suitable for commonly used packed bed chromatographic processes.

Simplified process design

Commonly used adsorption processes for purification of minor components have an advantage over liquid-liquid extraction if it comes to (toxic) solvent use. As shown in the previous section, it is possible to purify the isoflavones present in okara with few processing steps, and relatively environmental-friendly solvents (the used mechanisms this process is based on are shown in **Fig. 3c**). However, preparing an extract with fully dissolved solids and isoflavones is at the expense of a lower isoflavone yield, would need separate processing steps, generate extra waste (for changing the pH), and could possibly lead to further dilution. Therefore, we used a reversed approach, and instead of preparing an extract for packed bed adsorption, we prepared the adsorption bed suitable for use with the unclarified extract to do the extraction and adsorption in one step. This can be done, following a similar approach of Zhang et al. [32] who used expanded bed adsorption for the recovery of a target compound from a herb suspension.

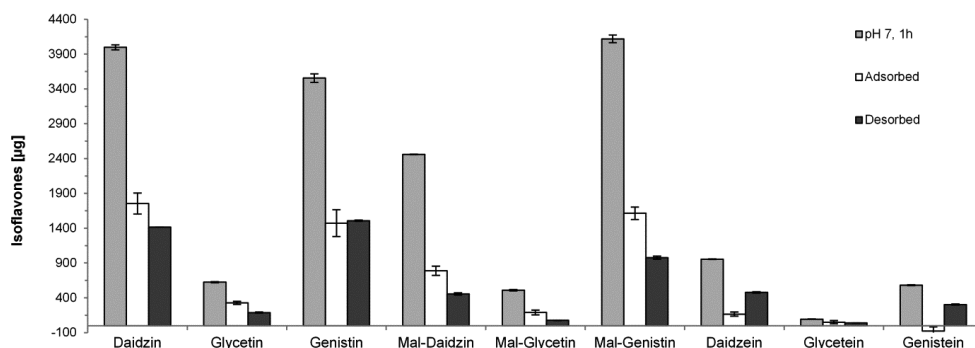


Fig. 4. Isoflavones [µg] in liquid before adsorption, calculated amount of isoflavones [µg] adsorbed, and isoflavones [µg] desorbed.

Fig. 4 shows the profile of isoflavones as extracted after 1h with water at neutral pH. It can be assumed that all malonyl-glucosides, and most glucosides were extracted with water, but part of the aglycones will remain in the matrix [27]. However, with the amount of resin used, around 45% of glucosides (42%, 56%, 42% of daidzin, glycetin, and genistin, respectively) and 35% malonyl-glucosides (32%, 36%, 39% of malonyl-daidzin, malonyl-glycetin, and malonyl-genistin, respectively) were adsorbed onto the resin. Most of these could desorb from the resin with 96% ethanol.

An interesting phenomenon was observed for the aglycones. The amount that was adsorbed onto the resin was calculated by the difference between the amount that was present after 1h

extraction (before resin addition) and the amount present in the solution after adsorption onto the resin. The amount of aglycones in solution after adsorption to the resin was either in the same range as before adsorption, or even slightly higher. This may indicate that either no aglycones adsorbed, or that further extraction from the matrix took place during to the adsorption. The presence of the aglycones in the desorbing solvent (**Fig. 4**) confirmed the latter; more aglycones were released from the matrix during adsorption, and around 52% of aglycones (54%, 45%, 57% of daidzein, glycitein, and genistein, respectively) were desorbed. Therefore, the aglycones have a higher affinity to the adsorbent than to the matrix. The conclusion from this experiment is that it is possible to combine the extraction and the isolation steps into one single step: this avoids problems (e.g. fouling of a packed bed column), and maximises the yield (by concurrent extraction). It should be mentioned that this is only possible by using a relatively poor solvent: a good solvent would not allow concurrent adsorption, as the isoflavones would remain in the solution. This experiment is a proof of concept that the mechanisms used in common unit operations can be used to make a complex system or process simple. Previously gained knowledge in this research [26, 27] about ionisation of the components and their different behaviour in water can now be used to optimise the process of a combined one-step separation.

At a low liquid-to-solid ratio, it should also be possible to extract all isoflavones, if simultaneous adsorption takes place; the solvent is only the carrier, but not the reservoir of the isoflavones. The already dissolved isoflavones adsorb, and the concentration in the water phase decreases, which allows further extraction, as is shown in **Fig. 4** with the aglycones. A similar approach has been recently suggested for the first time by D'Alessandro et al. [33]. In our approach though, the by-product does not need any pre-treatment, and the adsorbent is fully integrated in the extraction system.

Accurate purity measurements are difficult to achieve due to a low concentration of solids remaining in the ethanol solution for desorption, but taking samples from the desorbing solution from three independent experiments, centrifuging, and measuring solid content resulted in a purity of $7.4 \pm 1.6\%$, increasing the purity of the isoflavones almost 15 times in a single, un-optimised step. This shows that extraction and concentration is possible in one integrated simple step without many chemicals or resources. Such concentrations of isoflavones would already be acceptable for use as food ingredient, but the system has great potential for further improvement, and the purity can very likely be increased to a great extent. For example, an increased yield may be obtained with an elevated starting pH, following a low pH for adsorption; however, this is at the cost of a more complex process, and of the additional chemicals needed to adjust the pH. Secondly, 96% ethanol was used

here for the regeneration of the resin, but we expect that a lower ethanol concentration might be better. Thirdly, an increased yield due to a larger amount of resin or higher porosity will also positively influence the purity.

The configuration proposed here is a combination of the first three tasks of the task network shown in **Fig. 3b**. By avoiding thinking in unit operations or processing steps that have to be optimised in isolation, one can easier recognise where tasks can be combined and simplified. In this case, the separation process is altered by using an additional phase in the form of the resin, which is done in a form that does not require filtration. Based on the information gathered, this process can now be further optimised. As an example, the resin could be adapted to such process, i.e. it should be very porous for a high surface area, but should have a particle size that allows easy separation from the fibres. Alternatively, the resin particles can have a density that differs from the okara matrix to separate them by sedimentation. In addition, the previously gained detailed information on the traditional route will support the optimal design of the integrated extraction-adsorption. In the end, the revised task network comes very close to the first simple task network, which seemed unlikely at the beginning of the process design.

Evaluation of a strategic/holistic approach

As described in the section ‘the case study okara’, the different isoflavone forms show different behaviour, depending on their structure and the pH of the system. This and the previously shown advantages of using a holistic approach lead to the conclusion that the okara processing should not be seen in isolation from the soymilk production that precedes the okara processing. The fate of the isoflavones depends largely on the soymilk extraction process [34, 35]. Inactivation of the naturally present β -glucosidase, short processing times, and moderate temperatures prevent the hydrolysis of the naturally present glycosides of the soybeans into their respective aglycones or acetyl-forms. Due to the different behaviour of aglycones and glycosides, further process design depends on the ratio and presence of those components in the okara matrix. One could even design the process of the primary product (soymilk) such that the primary product keeps or improves its consumer acceptance and the by-product becomes more suitable for further processing and upgrading.

Thermodynamic understanding and quantitative models are crucial during process synthesis. In this case study, though, as in most food-related cases, okara has a highly structured matrix, of which the behaviour is difficult to predict due to the many components interacting with

each other. In addition, any external influence, be it pressure, temperature, pH, solvents, or other ingredients, can modify the behaviour of each component and their interactive relation. Simple models describing a complex system and summarising experimental data support the understanding of a complex system [27, 36].

The approach and effective solutions for a process synthesis problem can largely depend on the nature of the problem [9, 37] as also shown in this case. A separation process for ingredients requires a slightly different approach than building a food structure. The material structure and composition will determine the presence of thermodynamic (solubility or partitioning) or kinetic (diffusion) limitations, while tasks such as pasteurisation or mixing are less important. Adapting process synthesis methodologies from the chemical sector to the food industry will need further adaptation, and requires experimental input, which should possibly be captured in thermodynamic and kinetic models to predict the complex behaviour of the system. In case this is not possible, due to the structure and presence of multiple components in the by-product, it is possible to describe the behaviour with empirical models that give a relation between process conditions and extraction behaviour.

The collected experimental data and more detailed knowledge on the system enable the revision of the specific levels, and thus prevent the premature fixation of a flow sheet. The use of such methodology greatly supports the creation of additional possibilities and new concepts that can be further explored. Besides, it supports an open mind for unexpected steps in the process design. The creation of tasks, not unit operations, promotes the holistic approach and simplification of the process since it eases their combination. However, the assignment of building task networks and flow sheets requires a team of experts and creativity to apply the so-called heuristics.

Compared to process optimisation, process synthesis of a new process aiming at the development of new technologies is slightly more complex since not so many obvious heuristics may be present yet, which leads to the need of more experimental work. Nevertheless, such process synthesis methodology can facilitate the discovery of new process units, simpler processes, and thus, produces cheaper and more sustainable options. Besides, it directs future research questions to generate the relevant knowledge and point out the heuristics needed to complete the process design. The theoretical construction of task networks in combination with theoretical and empirical models and experimental experience with a system supports the non-narrowed (open-minded) thinking process.

CONCLUSIONS

Process synthesis methodologies are commonly used to design processes in the chemical industry systematically, but there is no clear strategy fully developed for the food industry. In this work, the strategic design of a new process for the production of isoflavone from the by-product okara is described.

The use of the process synthesis methodology allowed the generation of feasible alternatives for processing the okara with a focus on more sustainable processing. In this case, the traditional choice of an ethanol-water mixture for the extraction of isoflavones from the okara was shown to be suboptimal for the subsequent isolation step by affinity based adsorption of the isoflavones to a resin.

The traditional choice of an adsorptive system in the form of a packed bed was shown to be suboptimal as well, when it involves different solvents and necessitates extra polishing steps.

A combined, one-pot extraction and isolation (adsorption) step led not only to the elimination of these problems, but also to an increased extraction yield.

Thus, while the isolated consideration of each process step leads to choices that are not optimal in the next steps, considering the overall system on the level of general transformations, leads to different, globally optimal choices and in this case an even simplified processing system.

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Chapter 7

General discussion

RESEARCH AIM AND APPROACH

As described in the *General Introduction*, the by-product okara is produced in large quantities during the soymilk production. Okara contains amongst other components isoflavones, which belong to a larger group of polyphenols. The types of isoflavones in okara depend on the conditions during the soymilk production; generally, they are present in the form of malonyl-glucosides, β -glucosides, and aglycones. Polyphenols are considered to be valuable components due to their presumed health benefits. However, they are usually present in very small amounts in a complex matrix requiring efficient separation processes to isolate those ingredients. This thesis aimed to investigate the principles and mechanisms that underlie the separation of isoflavones from okara, and to evaluate the applicability of a process synthesis methodology to support the development of a sustainable and economic separation process for isoflavones from a complex matrix, based on these principles and mechanisms.

A combination of heuristics, a sustainability evaluation, and experimental and theoretical evaluations was used to draw the conclusions that will be discussed in this chapter. We will elaborate on the impact of other components in the matrix, answer the question whether it is possible to generalise the gained insight towards other polyphenol separation from different matrices, and conclude with future perspectives for more sustainable process design in the food industry.

MAIN FINDINGS AND CONCLUSIONS

Traditionally, separation processes to recover polyphenols comprise several steps such as pre-treatment of the material, separation of macromolecules such as protein, carbohydrates or lipids, extraction, and purification/isolation [1]. The purification of isoflavones often involves an extraction with organic polar solvents and subsequent chromatographic separation, sometimes in combination with membrane filtration, crystallisation, or several chromatographic steps making such processes complex for industrial application [2, 3]. Aqueous ethanol is increasingly popular as extraction solvent due to its status of being food grade and Generally Recognised As Safe (GRAS) [4]. Therefore, we investigated the extraction of the isoflavones in okara based on traditional principles, using a range of mixtures of ethanol and water in **chapter 2**. The polarity of the solvent was found to be the strongest parameter and mostly determined the distinction in behaviour between the different isoflavone forms. While the malonyl-glucosides could be almost fully solubilised with water, the aglycones solubilised best at high ethanol concentrations. It was determined that the

kinetics, the liquid-to-solid ratio, and temperature only influence the extraction at non-optimal polarity of the solvent, or if too little water was present in the system to sufficiently swell the matrix to allow for diffusive mass transfer.

To make the extraction process more energy and economically friendly, the pre-treatment of the material, including drying, was omitted, which yielded equally good results regarding the extraction yield. The use of the water originally present in the okara as part of the extraction solvent leads however to very high liquid-to-solid ratios if a high ethanol percentage is desired. Whether a drying unit or the liquid-to-solid ratio has more impact on the energy usage of these processes was analysed by mass and energy balances in **chapter 3**.

Analysing the extraction process further based on the first and second law of thermodynamics (exergy analysis) gave additional guidelines for sustainable process design regarding the use of resources. The glucosidic isoflavones are rather water-soluble, and the sustainability analysis combined with a conceptual process synthesis pointed out several aspects that would be of advantage if water were used as extraction solvent despite lower yields and purity at this stage. For example, the okara could be further used as animal feed without additional removal of the solvent; water as solvent does not require supplementation of the loss of solvent (ethanol), and a general high loss of exergy during the distillation that is needed for ethanol recovery can be avoided. For further purification, we studied the adsorption of the isoflavones dissolved in water-ethanol mixtures to commercially available adsorption resins [5]. Overall, the affinity of the isoflavones to the resins was highest when dissolved in water, which supported the hypothesis that extraction with water will lead to a more efficient overall process design.

Chapter 4 describes the specific behaviour of isoflavones in the diluted okara matrix in more detail. The lower solubility of the isoflavones in water compared to aqueous ethanol, and the co-extraction of other impurities into the solvent complicate the extraction. Nevertheless, all the glycosides could be efficiently extracted solely by increasing the ratio of water to solids, which suggests designing the extraction in stages or in a counter-current configuration. At high pH, also the aglycones could be solubilised in water, as well as the glycosides – all at low water-to-solid ratio. Their dissolution behaviour could partially be related to their molecular charges and thermodynamic behaviour. The largest drawback of water or alkaline water extraction is that proteins become co-suspended in the solvent, which makes the traditionally used packed-bed chromatography for isoflavone purification impossible. To study the adsorption from the water extract to resins commonly used for packed-bed technology, we lowered the pH of the extract to make most of the protein insoluble, and

separate it from the extract. This leads however to losses of isoflavones of up to 25%, likely due to association between the isoflavones and the proteins.

Chapter 5 describes the use of polyvinyl polypyrrolidone (PVPP) for the re-adsorption of the isoflavones from the solvent. The suspended proteins hardly affected the re-adsorption, except of course when the isoflavones were irreversibly lost with the precipitated proteins. Since the adsorption was also satisfactory without prior precipitation of the protein and with the entire okara matrix present, this precipitation step was found not needed anymore. Solubilisation of the aglycones was even possible without prior alkalisation if the extraction and adsorption were designed to act simultaneously. The underlying adsorption mechanisms were described with simple models using constant partition coefficients describing the affinity of the isoflavone groups towards the matrix and towards the resin (**chapter 4 and 5**).

A slightly up-scaled similar experiment was performed with a different resin in **chapter 6**, confirming the suggested mechanism. In the same chapter, the process synthesis methodology, which was further developed with this case, was described, supporting particularly the creative component of process design. The crucial level of defining the task networks (in contrast to unit operations) supported the unconventional combination of unit operations and allowed simplification of the process.

The matrix of okara, despite being considered a concentrated substance, is not more complex than the one of any other food product, and we have shown that existing technologies can be adapted to such concentrated systems. An improved mechanistic understanding of the behaviour of the system in combination with an exergy analysis has led to more sustainable process options in this work, and the elimination of energy-consuming processing steps will lead to a more economic and applicable process. The herein used process synthesis methodology required some extensions to be applicable for a separation process, but in general was found to be a useful tool to combine the various aspects of process and product design.

APPLICABILITY OF PROCESS SYNTHESIS

The design of a process by developing and investigating process steps separately, and solely on physicochemical considerations, was shown to be suboptimal: it would lead to the selection of a good solvent in regards to the isoflavones, which later in the process would generate the difficulty in finding an adsorption medium with sufficient affinity to re-adsorb the isoflavones, which would then require intensive regeneration procedures such as distillation and evaporation.

The process synthesis methodology we applied to this case was mainly developed for the design of structured food products, which means that different tasks become important within separation processes. For example, instead of mixing and stabilisation (sterilisation) tasks, separation tasks have to be defined. In addition, a separation/utilisation process deals with different issues than a structuring process. Instead of attributes that require the consumers' liking, solvent residues and separated fractions become important. Nevertheless, after some extensions, which are also further discussed in chapter 6, the methodology was found to be suitable for the synthesis of processing pathways for the separation of isoflavones from okara, and from this case study, we can derive a number of guidelines:

First, though considered as complex, the isolation of components from a concentrated matrix can be done using a relatively simple process such as extraction. Isoflavones, as many other polyphenols, are non-volatile components. Therefore, displacement from the matrix by bringing the components in a liquid phase seems to be required and reasonable. For a first estimation of the behaviour of the component, one can use the logP value (or partition coefficient), the structure, size, and number of hydroxyl groups, which can give an indication about the component's ability to interact with other components in the matrix, and its interaction/solubility in a solvent. One should keep in mind though, that polyphenols are ionisable components, and thus the logD value (distribution coefficient) may be more appropriate to use. For more volatile components such as vanillin or eugenol other mechanisms, such as evaporation or stripping, could be of more importance. In any case though, the process requirements defined in the framing level should be kept in mind, such as energy, water, and raw material efficiency. For example, high dilution of the system should be avoided.

Second, a concentrated solid matrix needs to be opened, which in general requires either swelling, or disruption of cells and cell walls, or both. The often semi-solid already processed matrix of by-products is in this case an advantage. Usually, the solvent should allow for some

swelling, which is however given for a high moisture by-product such as okara. If the matrix does not show much coherence though, as is the case for okara, the swelling may also induce suspension of part of the matrix in the solvent. This will require a subsequent separation later in the process, dependent on the extractant. However, we showed in this thesis that the process may be adapted to the suspended solids, such that it does not hinder in the re-adsorption.

Third, to be economically feasible, by-product utilisation requires complete separation into several valuable fractions or separation of one valuable fraction under the condition that the rest does not lose its former value. Particularly the latter implies mild treatments and exclusively natural processing aids, to preserve the by-product stream in its original state. We also found that the value of the by-product stream and the ability to process it can be influenced or improved by adaptations in the original process that produced the by-product stream. For example, optimisation of the soymilk production process to ensure the right composition of isoflavones in the okara would not greatly affect the soymilk properties, but would have impact on the isolation of the isoflavones from the okara.

Fourth, and somewhat counter-intuitively, one should use a solvent that is not too good, because this enables the combination of the extraction step and regeneration process based on affinity. The extraction requires a good solvent but the regeneration requires a poorer solvent. Consequently, an intermediate solvent quality is optimal to fulfil the requirements of the process.

Finally, if a system has suspended solids, do not use a packed bed column. Instead, a fluidised bed or an expanded column could be feasible. However, combining the extraction and the isolation, one overcomes several problems. First, the moderate solubility in the solvent is not a problem anymore, since it only acts as carrier. Second, the suspended solids from the okara do not interfere as they would do in a column. Third, the extraction of the okara can be enhanced. Nevertheless, this method introduces one extra step, namely the separation of the adsorption medium from the mixture. Designing this phase to be different to commercially available adsorbents in at least one property (e.g., density, size or perhaps incorporation of a magnetic core), would facilitate this step.

The latter and other aspects that require further discussion will be discussed in more detail in the following sections. The focus will be on the influence of specific interactions between matrix components and the target components (in our case proteins and isoflavones), and the properties of the matrix.

THE EFFECT OF INTERACTIONS BETWEEN PROTEINS AND ISOFLAVONES

Before the actual process design, we hypothesised that the interaction of isoflavones with components of the concentrated matrix may influence the process design: a large amount of literature reports the complexation of polyphenols with proteins [6]. However, very few attempts have been made to describe the possible specific interaction between isoflavones and soy protein thoroughly. The globular structure of soy protein makes it a poor candidate for specific binding to the polyphenols [7]. However, the soy proteins glycinin and β -conglycinin contain enough proline residues on their surface to bind polyphenols potentially [8]. The denaturation state of protein in okara is thought to increase its ability to interact with polyphenols, since more hydrophobic parts get exposed. However, the polyphenols' structure, conformation and flexibility are as important. A high molecular weight, high flexibility, and low water-solubility increase the capacity of the polyphenol to bind to proteins [6, 7]. Hence, the better water solubility of the glycosidic isoflavones implies a lower ability to bind to proteins, whereas the aglycones will show much larger affinity for protein/polyphenol complexes, even though their molecular weight is rather small with less than four aromatic rings.

The results presented in **chapter 4 and 5** indicate a high affinity of the aglycones to other components in the system. At higher liquid-to-solid ratios, a further increase of the ratio did not lead to any more isoflavones extracted, although the concentration that can be extracted with ethanol was not reached. This was attributed to the ethanol being able to weaken the protein-polyphenol interactions [6] (**chapter 2**) and that the aglycones' solubility is much greater in ethanol than in water [9]. Further insights of the interaction between the proteins and isoflavones could be established during ultrafiltration trials. The protein could be separated from the aqueous extract by a regenerated cellulose membrane with an Amicon cell at pH 10, whereas at pH 7 this separation was not successful, leading to less aglycones recovered in the permeate solution and a larger amount that could not be accounted for. It can be hypothesised that both the protein and the isoflavones are negatively charged at high pH, which is likely not the case at neutral pH for the aglycones.

This is in line with the findings in **chapter 5**, where the extracted protein was precipitated and a reduction of up to 25% of isoflavones was found in the supernatant. A clear preference of the aglycones to complexate and precipitate with the protein is shown, with genistein having the highest reduction followed by daidzein.

The interaction between proteins and polyphenols is often described as driven by hydrophobic interactions further supported by hydrogen bonds [6]; however, the precise mechanism depends on the medium, in which the interaction takes place. The solvent polarity, pH, and temperature are some parameters that can influence the interactions, which explains the slightly different findings of different researchers investigating the isoflavone rich protein isolates [10-14], especially regarding the amount of isoflavones that precipitates with the protein.

UNDERSTANDING THE MATRIX EFFECT ON EXTRACTABILITY

To investigate the system of interest in the most conventional way, we extracted the isoflavones from okara with different solvents, and used a good solvent (50% ethanol) to test the influence of the liquid-to-solid ratio, and temperature, which both did not show much impact. Typically, the samples to extract are freeze-dried, which we did to examine whether kinetic limitations during the extraction process exist. The results shown in **Fig. 1** are not discussed in previous chapters, but revealed an important mechanism.

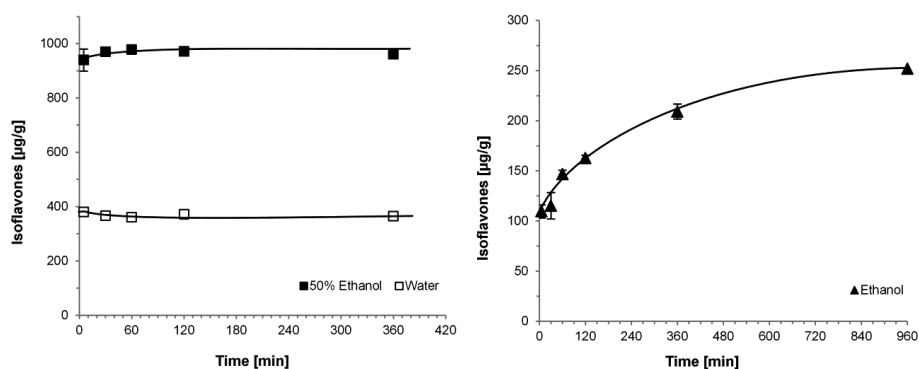


Fig. 1. Extraction kinetics for a) 50% ethanol, and water as solvents, and b) ethanol as solvent (extraction at room temperature, liquid-to-solid ratio 10 to 1). Lines are represented to guide the eye.

The freeze-dried okara was extracted with water, 50% ethanol, and pure ethanol. Extractions were done between 5 min and 16 h at room temperature. A kinetic effect for those extractions is only observed with pure ethanol (**Fig. 1**), which implies that water is essential to swell the matrix and increase the diffusion rates of solvent and isoflavones to practical values. With pure ethanol, the isoflavones are extracted only very slowly. Extraction with water does result in a lower yield, mostly due to low solubility of some isoflavones (e.g. the aglycones), but for both water and 50% ethanol, no kinetic limitation is seen. Okara consists of the insoluble fibrous residue. Besides the cell walls, consisting of cellulose and hemicellulose, it retains some proteins and other components. This residue swells strongly in water, but not in ethanol.

During the process used in this research, around 80% of the isoflavones partitioned into the soymilk, and the other 20% remain in the okara (at 105 °C) (**Fig. 2**). Previously, researchers found 12% to 30% of the isoflavones originally in the soybeans in the okara [15, 16]. The majority of the isoflavones that are extracted from okara using water or 50% ethanol seems to stem from the soymilk residue that is absorbed in the fibres after separation of the ‘liquid’ (soymilk) and ‘solids’ (okara) during the soymilk production process.

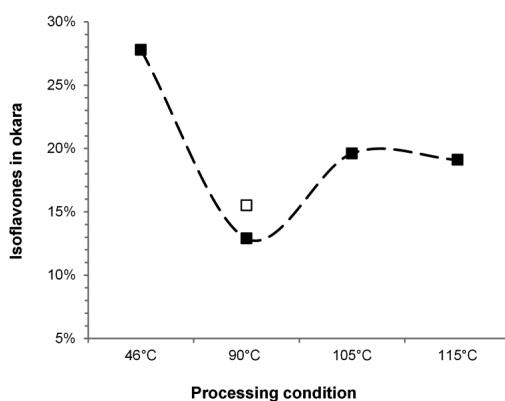


Fig. 2. Isoflavones in okara [%] as function of processing temperature and time in respect to the amount in the starting material (soybeans). The okara was processed with an ASC50 soymilk system as described in chapters 2 and 4. The cooking time was 3 min, except for 90 °C, where the cooking time was as short as possible (~0 min) (filled marker), and 30 min (empty marker). The line is represented to guide the eye.

The remaining part is not soluble in the solvent, and will not show dissolution in time. This part is more hydrophobic, and can be extracted better with a less polar solvent mixture. This part depends on the processing history. At 46 °C for example, the okara sample contains a high amount of aglycones, due to β -glucosidase activity [17, 18]. Thus, such okara will show low extraction levels with water, but considerably higher levels with a 50%-80% aqueous ethanol mixture. At higher temperatures during soymilk production, the β -glucosidase is inactivated, but the glycosides can be thermally hydrolysed.

A short soymilk extraction process at 90 °C gives the lowest thermal and enzymatic hydrolysis and thus the highest water extractability, while a longer processing time converts more malonyl-glucosides into β -glucosides, which are less water-soluble than the malonyl derivatives. Processing at higher temperatures slightly increased the amount of aglycones on the expense of β -glucosides, and malonyl-glucosides transform into β -glucosides. Thus, the short soymilk extraction at 90°C will give the least isoflavones in the okara, since most of it is already extracted into the soymilk (with a residue remaining in the okara), while the okara from a longer, a low-temperature or a high-temperature process will give more isoflavones in the okara, which can then be extracted with a water/ethanol mixture.

OVERCOMING THE MATRIX EFFECT

During the soymilk extraction from the soybeans, the aglycones show already a high affinity to the matrix and a large part remains in the okara. In **chapter 4**, we described the high affinity of the aglycones to the matrix. A stronger solvent (with lower polarity than water) is necessary for the partitioning of the aglycones towards the solvent, and additionally leads to higher selectivity (**chapter 2**), which is the traditional approach. Isoflavones, and more general flavonoids, have commonly been extracted with organic solvent [4, 19].

More recently though, the focus has been on extraction techniques that are more environmentally benign and can overcome some of the limitations such as large amounts of organic solvents, thermo labile components, or low selectivity, e.g. ultrasound-, microwave-, or enzyme-assisted extraction, supercritical fluid extraction, and pulsed electrical fields. Also pressurised hot water extraction has been investigated for flavonoids showing that if the dielectric constant of water is decreased by the high pressures, many flavonoids can solubilise in the subcritical water [20, 21]. However, this technology requires relatively stable components. The use of a strong solvent for the extraction will by definition need a strong driving force for isolating the isoflavones from the solvent, for example by evaporation, and

in many cases, adsorption on a resin will not be possible, since the components dissolve so well in the solvent, that they will not adsorb.

With the help of the process synthesis methodology (**chapter 6**), we suggest a different approach in this thesis. As described previously, the low affinity of the aglycones to water (high relative affinity to the matrix) can also be overcome either by extraction at high pH, or by adding a third phase (besides the matrix and the solvent), which, if added to the mixture, leads to a concentration drop in the liquid that allows further solubilisation in the liquid. Both approaches lead to very large savings in energy, resources, and costs. On the one hand, a large amount of organic solvent can be spared in the first step, which reduces costs and the footprint implied by its recovery, and loss to the environment (**chapter 3**), and on the other hand, the extract can be immediately further processed with existing technologies such as affinity chromatography without evaporation of the solvent [5].

To conclude, by designing the process as a whole, and not step-by-step, we obtain different designs. The optimisation of the extraction step only, leads to the selection of a good solvent to extract the highest yield of isoflavones in a simple way. However, simultaneous optimisation of the extraction and the adsorption step, and environmental restrictions defined in the framing level lead to the selection of a moderate solvent that allows both some solubilisation but also concurrent adsorption onto a resin, in effect only using the solvent as transfer medium.

APPLICATION TO OTHER FOOD BY-PRODUCTS

By combining the understanding of the interactions of the components with the media used (solvents and extractants), with a process synthesis that starts with the whole process instead of individual steps, we were able to identify a more efficient process route.

A complex system does not always need complicated processing. In the case of isoflavone extraction from okara, the main issue is the opening of the matrix to allow for the extraction of the isoflavones, and the selection of the right solvent to allow efficient removal and isolation of the isoflavones. The soybean matrix is disrupted during the production of the soy slurry by grinding, which allows a relatively simple first extraction, rather similar to the initial production of the soymilk.

Certainly not all by-products have a similar open structure with some readily available components in it, but a good example of a product similar to okara is brewer's spent grain (BSG). BSG is the by-product of the beer production arising at the lautering step after malting and mashing the grains and separating the wort (liquid) from the grains (solids). This leads to a solid-like product with a moisture content around 80% which contains a considerable amount of polyphenols [22-24]. An investigation of extraction solvents for polyphenols from BSG [22] demonstrated that their affinity to several solvents is similar the affinity of soy isoflavones to solvents investigated for isoflavones. Therefore, the simultaneous extraction-adsorption process suggested in this thesis could be a possible processing route to recover, and purify the polyphenols present in BSG in a sustainable and efficient way as well.

Another example is the recovery of flavonoids from onion waste. Residues and surpluses of onions are very rich in flavonols. The most abundant and well-studied flavonol is quercetin, present mainly as glycosides in onions, but also as the aglycone in the waste stream [25, 26]. Commonly, the two main steps of extraction and purification are investigated separately as also exemplified in the case of onion waste [26-28]. However, following the outcome of this research suggests that a combination of those processing steps could potentially lead to a better option.

Apple pomace is an example, in which a pre-treatment may have to be considered. Virot et al. [29] studied the effectiveness of ultrasound on the matrix and the extraction of its polyphenolic components. They concluded that ultrasound had more impact on the components that were originally present in the peel of the fruit than on the polyphenols elsewhere located. Ultrasound increased the yield by around 20% for an extract with aqueous ethanol [29] and by around 30% for a water extract [30]. Any polyphenols that are still located in intact cells of the fruit's peel may be difficult to extract. The majority of the phenolic compounds present in apple pomace are nonetheless likely to be extracted more efficiently with the suggested mechanisms. The authors reported the same bell-shaped curve for the total phenolic compounds at different ethanol-water mixtures as reported for the total isoflavones in **chapter 2**. For further purification of the components it is then suggested to use a solvent of moderate polarity, in which the components are however partially soluble (e.g. water) [30]. A moderate solvent creates a high activity of the component, which leads to easier adsorption to introduced particles, such as affinity beads.

Our approach described in this chapter is similar to some biorefinery concepts, where the by-products are given value by extraction of valuable chemicals/phytochemicals using environmentally benign technologies [31].

From a different perspective, one could even decide to extract the isoflavones from soymilk by simply contacting the soymilk itself, during extraction, to a resin, which will then adsorb the isoflavones, while leaving the other components in the soymilk. The removal of the isoflavones during the soymilk production with the help of beads with specific affinity may lead to an improved taste of the soymilk, because the soy aglycones have shown to activate certain bitter receptors [32]. At the same time, this configuration will result in a much higher overall yield of isoflavones in the residue.

STRATEGIES FOR MORE SUSTAINABLE SEPARATION PROCESSES

The interest in extracting and purifying bioactive components from plants and food matrices has drastically increased. Research efforts focus commonly either on the ‘ideal’ extraction methods and solvents (i.e., disregarding the isolation step), or the purification of components with chromatographic systems or membranes. More recently, the focus has shifted towards milder solvents, and more energy-efficient technologies to overcome the limitations imposed by toxic solvents, instable components, but most importantly, to fulfil the ever stricter regulations to produce safe and healthy food products in a sustainable way. However, systematic approaches to conceptually design the recovery of high value components from a food matrix are rare. Many aspects of our conceptual design approach have been discussed in **chapter 6**. We based our approach on the recently developed product-driven process synthesis methodology [33], which is based on a hierarchical approach including the complexity of a structured food product. The use of this methodology resulted in two main process routes (**Fig. 3**), which remain to be studied in further detail.

Both process routes resulted from the investigation of underlying mechanisms and properties of the system and the desired components accompanied by exergy analysis (**chapters 2 to 5**). Process A has still similarities to more conventional approaches, where the steps of extraction and purification are seen as separate steps. One should realise that the protein precipitation step is only needed because of the limitation of the next processing step: the packed bed does not allow for suspended solids or easily adsorbing components such as proteins.

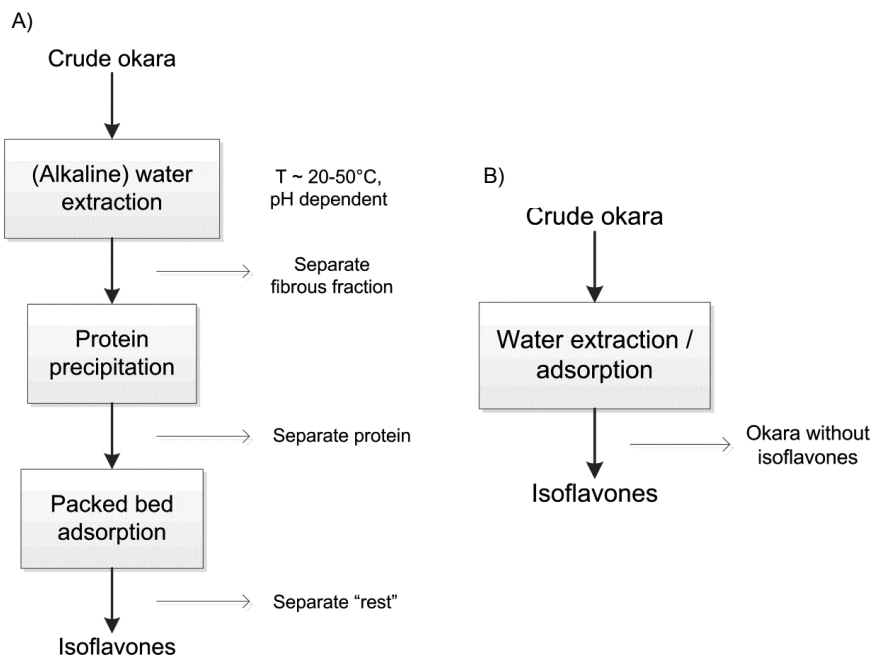


Fig. 3. Two base-case process routes for the separation of isoflavones from okara

Generally, the steps are separately optimised, involve several extra preparation steps, and a holistic view on the process system and investigation of mechanisms allowed us to integrate these processing steps better [5] (**chapter 6**). However, the constant revision of the task networks led to the combination of the tasks of separation of fibres (insolubles) and separation of isoflavones.

Investigations in **chapter 5 and 6** showed that the system could indeed be designed with a simultaneous extraction – adsorption, which is similar to processes suggested by Zhang et al. [34] and D’Alessandro [35] for integration of extraction with simulated bed and packed bed technology, respectively. In our system, the resin was directly added to the slightly diluted okara matrix. We kept the resin separated from the matrix by a sieve. However, if the particles could be mixed better with the matrix, mass transfer would be increased. Separation of the particles could be achieved by introducing particles that are much bigger to allow separation

by size, that have a heavy core, which would allow separation by sedimentation, or they should be designed with a magnetic core to be separated based on magnetic principles. In fact, magnetic beads as magnetic adsorbents are a promising tool in bioseparations, e.g., to separate proteins from crude feedstock [36, 37].

Cerón et al. [38] approached the recovery of polyphenols from a fruit (*Matisia cordata*) based on a superstructure and computer-aided design. While each unit operation is treated as a step itself, the design space is limited. This case study clearly proved the advantage of the PDPS approach. While the isolated consideration of each process step leads to choices that are not optimal in the next steps, considering the overall system on the level of general transformations (tasks), leads to different, globally optimal choices and a simplified processing system. Furthermore, it supports the inclusion of non-conventional innovative processing steps or combinations as opposed to mathematical superstructures or optimisation of existing unit operations.

Nevertheless, while starting with a methodology designed to make structured food products for a separation process, we experienced several drawbacks that were not considered in the initial methodology. Defining the output was the first challenge. In case of a structured product, the outcome is defined in terms of structure, taste, and colour defined by consumer wishes. The outcome of separation process is not clear, difficult to predict and strongly dependent on a range of factors. One major example is the consideration of resulting side products if a pure isoflavone isolate is the original desired output. With prerequisites of avoiding many processing aids, toxic solvents, considering least energy use, and no waste of resources, the process design has to become rather ‘application-driven’ than ‘product-driven’, which automatically integrates the objective of sustainability.

This is in agreement with the emerging biorefinery concept, where each fraction is considered as product and the concept of low-value by-products is not accepted. Previously, process design approaches in the food industry have been inspired by the concepts of chemical engineering as summarised in [39] and **chapter 6**. Now we seem to have reached an intercept of the two disciplines, since process synthesis has recently also found application for biorefinery processes [40], which also inspire academics and industry of the food supply chain.

As mentioned previously, the application-driven approach seems appropriate for separation processes. The price, the product, and sustainability (efficiency) are essentially objectives within process synthesis that are interrelated, and depending on the era, we have given them

different significance. Environmental sustainability is not the only aspect of a sustainable way of life and needs to be combined with economic and social sustainability [41], which can be translated to the objectives of environmentally benign processes, which are at the same time economic, and lead to outputs that are acceptable by the consumer. Design methodologies are in development for the food industry, and it may be of advantage to combine aspects of both the hierarchical and mathematical approach, and for instance use a pareto optimisation in a later stage of the process design.

Sustainable process design is included in more and more design approaches [40-42]. In this work, we show how the sustainability assessment tool based on exergy analysis can be incorporated in the process design (**chapter 3**). By including exergy analysis, sustainability (which means optimisation of several sub-objectives in an objective manner, not just energy consumption) can be included. In many cases, a more exergy efficient process is also more economic due to savings in water, energy and resources. Nevertheless, the economics depend primarily on the recovery (yield) of those high value components, which we included in our model in **chapter 3**. The assessment of design options with exergy analysis was found to be a useful tool within sustainability oriented process design, and future process synthesis methodologies for the food industry should integrate exergy in the design method.

Provided that the okara resulting from the soymilk production consists mainly of glucosides, increasing the pH is not necessary, unless less dilution of the system is desired (**Fig. 3 a**). In several steps of the process design, a distinction has to be made between the aglycones, which are less water-soluble and interact more with the matrix, and the glucosides, which are less stable, but naturally present in the soybeans. If a high percentage of aglycones is present in the material, one might even consider a more traditional route using ethanol as extraction solvent at first.

Making this distinction between the different isoflavone forms in the process design, leads to the conclusion that the original process, in this case the soymilk production, should also be integrated in the process design. Either one can optimise the pre-processing (in our case the soymilk production) to give the isoflavone profile best for a certain process design, or the okara can be better integrated in the process. A focus on a natural isoflavone profile (i.e. the glucosidic forms), would on the one hand lead to less isoflavones to start with in the okara, but on the other hand it would lead to milder processing steps, because they are easier extracted with water than the aglycones. Furthermore, the aglycones have more potential to interact with other components in a matrix, which complicates the processing, and they have an impact on the taste of the soy product [32]. Therefore, a focus on the glucosides would

lead to a simple and natural method to extract them, while the resulting product will be less bitter and better accepted by the consumers.

The reduction of chemical waste has been a first step, followed by an increasing effort to fully utilise by-products from the food industry. Increasing the value of by-products is only a transitional solution for more sustainable processing. The next step requires optimising even larger parts of the processing chain holistically through optimising the processes leading to the so-called by-products for production of other ingredients, as shown in the example of the soymilk production. Side streams have to be re-considered as valuable products, and integrated biorefinery concepts will then lead to an even more efficient food supply chain.

CONCLUSION

To arrive to more efficient separation processes for polyphenols, the matrix has to be studied to estimate whether additional processing steps are necessary to create an open structure (e.g. with ultrasound, pulsed electrical field, or grinding). It was shown that the matrix as such only plays a minor role compared to the improvement that can be made in sustainability of the system and process efficiency. It is possible to simplify processing steps, integrate extraction and adsorption, and purify the isoflavones with an easy processing step, and interaction of the molecules with the matrix can be overcome by smartly modifying the processing conditions.

For other concentrated systems those mechanisms may work as well, but will have to be adapted to each specific case, and it will stay indispensable to investigate the extent of interaction of the desired component with its matrix. A large amount of data can be found on polyphenols, their properties and adsorption behaviour. Nonetheless, most data is found about the components in pure solvent systems, and it is suggested to direct research towards the direct investigation of complex systems for the adaption of processes in the food industry. Solubility parameters or $\log P/\log D$ values can be used for first estimations about the behaviour of the polyphenol in a solvent. However, their large number of complex structures complicates predictions and their stabilities and interaction with the matrix has to be studied nonetheless.

Screening of the solubility of the desired component in solvents, followed by a counter-intuitively choice of a bad solvent (e.g. water) to create a high activity, should enable the simultaneous adsorption of the component on an additional phase, which again leads to large energy and resource savings. However, not only the process for recovery of valuable

components should be better integrated, but primary production processes should also be considered.

The process synthesis methodology supported the holistic design approach, but had some drawbacks in the use for a separation process. Finally, the sustainability assessment should receive more attention by getting fully integrated in the design method.

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Summary

Samenvatting

Resumen

By-product utilisation, more efficient use of resources, and more sustainable processing have become of the utmost importance for society and the food industry. During soymilk production, a by-product called okara is produced in great quantities. Despite being a by-product, okara contains many nutrients, which could be utilised for human consumption. Isoflavones are one example of the components present in soy, which are also found in okara. Isoflavones are a subclass of flavonoids, a group of the many polyphenols that exist, and are believed to have a positive effect in the prevention of hormone related cancers, cardiovascular disease, osteoporosis and obesity. One of the main challenges when extracting isoflavones from okara are their low concentration in okara and the strong water binding capacity of the biopolymer matrix of okara. Besides, isoflavones comprise several classes of components having different properties with respect to for example their solubility. This makes the separation and purification of those components in a cost-efficient manner with a non-toxic solvent route challenging.

The aim of this thesis is to provide insight for the development of a polyphenol separation process on the case of okara. The presence of a partly solid matrix and its impact on the separation process was investigated, and the opportunities and challenges for isoflavone separation from okara are presented in this thesis. By means of this case, the applicability of a process synthesis methodology for separation processes was investigated.

In **chapter 2**, the use of ethanol and water was investigated as relatively non-toxic solvents for extraction of the isoflavones. High extraction yields were obtained with 50-70% ethanol. However, different optima in the range of 0%-100% ethanol were obtained dependent on the isoflavone group, which highly differ in hydrophobicity. The glucosides had an optimum at 50%-60% ethanol, the malonyl-glucosides between 30% and 60% ethanol, and the aglycone yield increased monotonously with the ethanol concentration. The solvent choice does not just determine the yield of isoflavones in the extract, but also the swelling of the matrix, important for the separation of the solvent and the okara, and the purity in the extract. While finding a good solvent for a range of components is one issue, a second challenge in the utilisation of okara is its high moisture content, because this limits the ethanol concentrations that can be used in the extraction solvent when extracting the crude okara. Nevertheless, the same extraction yields were obtained as with the dried material, which led to the conclusion that drying of the starting material, being an energy intensive operation, should be omitted.

The consequences of using the wet, non-dried okara were evaluated using an exergy analysis. The use of the water in okara poses a challenge if a high fraction of a co-solvent such as ethanol is needed, since that increases the total liquid-to solid ratio. Therefore, we

conceptually designed the extraction process and evaluated the efficiency and sustainability of different options (**chapter 3**). Exergy analysis can quantify and combine effects of solvent consumption and physical energy thermodynamically. In addition, it can indicate the resource efficiency of a process and can be used to compare streams with different solvents such as ethanol and water. Often used for process optimisation, we investigated the use of exergy for process synthesis, which delivered the information for further process design and decision-making. A drying step was found to be less detrimental than an increased solvent use. The use of ethanol, its loss and distillation, and the loss of the extracted residue were identified as the most inefficient steps within the conceptual process, and with this, guidelines were given how to improve the systems sustainability.

The analysis performed in chapter 3 showed that a significant step towards better sustainability is made when ethanol is fully omitted as solvent in the extraction step. Furthermore, it was shown in chapter 2 that part of the isoflavones are rather water soluble due to their glucosidic nature. Therefore, the extraction and solubilisation of isoflavones in water was investigated in detail in **chapter 4**. Besides, the co-extraction of proteins in the water environment and their effect on the isoflavone extraction was investigated. The temperature did not influence the extraction, but an increased liquid-to-solid ratio and the pH did have a clear influence on the extraction yield. Okara may also contain the less polar aglycones, which are in general not present naturally in the soybeans, but which are products of hydrolysis of the glycosides due to certain processing conditions. The ionisation (dissociation) of the components at higher pH can modify their solubility and interaction with the matrix; therefore, by adjusting the pH the aglycones could be solubilised in water as well.

Further purification based on affinity separation was shown to be more feasible with water as solvent, since a water-ethanol mixture would be such a good solvent that the adsorption on the column would be hindered. The common way of regenerating such components includes the use of another solvent than is used for extraction, leading to an additional evaporation step. The higher affinity of isoflavones in an aqueous environment to a resin, and the possibility to omit an additional preparation step demonstrated the suitability of water as solvent in the primary extraction step: the extracts are more suitable for chromatographic purification, while ethanol or aqueous ethanol could be used as eluent. This demonstrates that it is important not to design a processing step by itself, but to optimise an internally coherent processing system as a whole.

Chapter 5 reports on the adsorption of the isoflavones on polyvinyl polypyrrolidone (PVPP) with special regards to the effect of proteins that are co-suspended in the extract. The

adsorption efficiency was only negatively influenced when accounting for the isoflavones that are lost with protein that precipitates at lower pH. The affinity of the isoflavone groups could be described with constant partition coefficients resulting from a simple model. Furthermore, the adsorption of the isoflavones onto PVPP with the entire okara matrix present at floating pH could be described with a model using the partition coefficients describing the affinity to the PVPP and to the matrix. The model suggests that it is possible to ‘pull’ the isoflavones from the okara by concurrent adsorption of the isoflavones to an adsorption resin that is present in the same solution, thus increasing both yield and purity.

The three main isoflavone groups present in okara behave differently throughout the entire study, due to their different properties. The order of their adsorption affinities was the reverse of their water extractability. A more sustainable integrated recovery process can be designed for polyphenolic components, by identifying the right balance between the polarity and hydrophobicity of the component of interest, the solvent, and the adsorbent.

The investigated process synthesis methodology, and the design options that followed by applying this methodology are discussed in detail in **chapter 6**. A simplified simultaneous extraction-adsorption separation process, which resulted from the process synthesis is presented. Two base case flow diagrams that can be investigated in depths are further discussed in **chapter 7**.

Since the relevant properties and phenomena underlying the results presented in this thesis are quite generic, they can be translated to other separation processes, and the same approach can be used to develop more sustainable separation processes for polyphenols or other useful compounds from other food-by-products. By using the sustainability and efficiency as a prime objective within process design in combination with a good understanding of the underlying phenomena of the system, a new step in process design can be made: Sustainable process development requires proper understanding of the complex systems we are dealing with, the matrix itself, but also the thermodynamic behaviour of the components, and their behaviour in the matrix and during processing.

Summary

Samenvatting

Resumen

Het beter valoriseren van bijproducten en een efficiënter gebruik en verwerking van grondstoffen zijn van groot belang voor de maatschappij en de levensmiddelenindustrie. Bij de productie van sojamelk wordt een grote hoeveelheid bijproduct genaamd okara gevormd. Okara is rijk aan nutriënten die geschikt kunnen zijn voor humane consumptie. Een voorbeeld van zo'n nutriënt in okara is de groep van isoflavonen. Isoflavonen behoren tot de categorie van de flavonoïden; flavonoïden zijn polyfenolen waaraan positieve gezondheidseffecten worden toegeschreven, met name in het voorkomen van hormoon-gerelateerde kankers, hart- en vaatziekten, osteoporose en obesitas. Een van de voornaamste uitdagingen in de extractie van isoflavonen uit okara, is de lage concentratie van deze componenten in okara en het grote waterbindend vermogen van de matrix waaruit okara bestaat. Daarnaast bestaan isoflavonen uit verschillende componenten die weer verschillende eigenschappen hebben, zoals de oplosbaarheid. Dit maakt het winnen en verder zuiveren van deze componenten op een kosteneffectieve manier zonder gebruik te maken van giftige oplosmiddelen niet eenvoudig.

Het doel van dit proefschrift is het vergroten van de kennis die nodig is om een winningsproces van polyfenolen uit okara te ontwikkelen. De aanwezigheid van een deels vaste matrix en de gevolgen daarvan voor het winningsproces zijn onderzocht. De mogelijkheden van en de uitdagingen in de extractie van isoflavonen worden ook beschreven in dit proefschrift. Dit proces is verder gebruikt om de toepasbaarheid van processynthese voor winningsprocessen te evalueren.

Hoofdstuk 2 beschrijft het gebruik van ethanol en water als relatief niet-toxische oplosmiddelen voor de extractie van isoflavonen. Hoge opbrengsten werden verkregen met 50-70% ethanol. De exacte opbrengst varieerde per component vanwege verschillen in hydrofobiciteit, als gevolg waarvan de optimale concentratie van ethanol niet voor elke component hetzelfde was. De glucoside-vorm gaf optimale extractie bij 50%-60% ethanol en de malonyl-glucoside bij 30%-60% ethanol. De opbrengst van de aglycon-vorm nam toe bij hogere ethanolconcentratie over het hele concentratiegebied. Het oplosmiddel bepaalde niet alleen de opbrengst, maar ook het zwelgedrag van de matrix, wat de scheiding van oplosmiddel en okara beïnvloedde en ook de zuiverheid van het extract beïnvloedde. Als gevolg hiervan is het vinden van een goed oplosmiddel voor de componenten één uitdaging, en een tweede uitdaging is het gebruik van niet-gedroogde okara. Het hoge watergehalte van niet-gedroogde okara beperkte namelijk de maximaal haalbare ethanolconcentratie bij een gegeven hoeveelheid oplosmiddel. Daarom is ook het effect van drogen van okara op de extractie onderzocht. Het bleek dat drogen geen invloed had op de opbrengst van de extractie. Omdat drogen een energie-intensief proces is, moet deze stap bij voorkeur achterwege gelaten worden.

Een exergie-analyse is uitgevoerd om de verschillende extractiemogelijkheden te vergelijken. Er is vooral gekeken naar de mogelijke voordelen die het gebruik van niet-gedroogde okara biedt ten opzichte van gedroogde okara. Het niet drogen van okara kan leiden tot het gebruik van meer oplosmiddel om de juiste ethanolconcentratie te bereiken. Daarom zijn verschillende processen geëvalueerd op efficiëntie en duurzaamheid en de uitkomsten zijn beschreven in **hoofdstuk 3**. Er is gekozen voor een exergie-analyse omdat met deze analyse de effecten van oplosmiddelgebruik en fysische energie gekwantificeerd en vergeleken kunnen worden. De exergie-analyse beschrijft de efficiëntie van grondstofgebruik en vergelijkt stromen van verschillende samenstelling. Een droogstap was minder nadelig dan het gebruik van meer oplosmiddel. Het gebruik van ethanol, het verlies en de distillatie van ethanol en het verlies van materiaal werden als meest inefficiënte stappen geïdentificeerd. Verder is gekeken hoe uitkomsten van de exergie-analyse kunnen dienen als input voor de processynthese.

Uit de exergy-analyse beschreven in hoofdstuk 3 bleek dat het gebruik van ethanol als oplosmiddel vermeden moet worden. Voor een duurzaam extractieproces is het beter om water als oplosmiddel te gebruiken. Dit lijkt mogelijk omdat een deel van de isoflavonen water oplosbaar is (hoofdstuk 2). Om deze redenen is het gebruik van water als extractiemiddel verder onderzocht in **hoofdstuk 4**. Het bleek dat temperatuur de extractie van isoflavonen niet beïnvloedde, maar het gebruik van meer water leidde wel tot hogere opbrengsten van met name de minder polaire aglyconen. Daarnaast is de co-extractie van eiwitten en het effect hiervan op de extractie van isoflavonen onderzocht. De pH bleek een goede parameter om de zuiverheid van het extract te verhogen. Een hogere pH leidt tot ionisatie van de componenten en een vermindering van de interactie met de matrix; de aglyconen bleken beter oplosbaar in water bij verandering van de pH.

Verdere zuivering door middel van een scheidingsproces gebaseerd op affiniteitsverschillen tot een bepaald resin was ook beter mogelijk wanneer water als oplosmiddel werd toegepast. Een voor de extractie beter oplosmiddel dat ethanol bevat, hindert de adsorptie te veel. Bovendien is voor de desorptie van de componenten een ander oplosmiddel nodig dan het primaire oplosmiddel, waardoor een extra verdampingsstap nodig is. De hogere resinaffiniteit van isoflavonen in water en de mogelijkheid om een extra processtap achterwege te laten zijn verdere voordelen van het gebruik van water tijdens de primaire extractie. De waterige extracten zijn zeer geschikt voor chromatografische zuivering, waarbij een ethanol-water mengsel als elutievloeistof kan dienen. Bovenstaande resultaten laten zien dat het belangrijk is om een gehele procesketen te analyseren en optimaliseren en dat optimalisatie van afzonderlijke processtappen niet altijd leidt tot het meest optimale proces.

Hoofdstuk 5 beschrijft de adsorptie van isoflavonen aan polyvinyl polypyrrolidon (PVPP), waarbij de focus lag op de rol van de eiwitten in het extract. De adsorptie efficiëntie werd alleen negatief beïnvloed doordat bij lage pH isoflavonen neerslaan met het eiwit. De affiniteit van de isoflavonen tot PVPP kon worden beschreven met een eenvoudig model met constante verdelingscoëfficiënten. De adsorptie van isoflavonen aan PVPP in de aanwezigheid van okara waarbij de pH niet gecontroleerd werd liet ander gedrag zijn. Dit gedrag kon gevat worden in een vergelijkbaar model met verdelingscoëfficiënten die de affiniteit van isoflavonen tot PVPP en de okara-matrix beschreven. Modelberekeningen toonden aan dat het mogelijk is om de isoflavonen uit de matrix te “trekken” door de gelijktijdige adsorptie van isoflavonen aan de resin aanwezig in dezelfde oplossing. Op deze wijze was het mogelijk om opbrengst en zuiverheid te vergroten.

De drie groepen isoflavonen die het meest voorkomen in okara vertoonden verschillend gedrag in alle experimenten verricht in dit onderzoek. De mate van absorptie van deze groepen aan PVPP nam toe met afnemende wateroplosbaarheid. Een (duurzaam,) geïntegreerd winningsproces voor polyfenolachtige componenten kan worden ontworpen door te kiezen voor een juiste balans van polariteit en hydrofobiciteit van de te winnen componenten, het oplosmiddel en het absorptiemateriaal.

De onderzochte methode van processynthese en de daaruit volgende ontwerp mogelijkheden worden in detail besproken in **hoofdstuk 6**. Het toepassen van de ontwerp methode leidde tot een vereenvoudigd, gecombineerd extractie-adsorptieproces. In **hoofdstuk 7** worden twee procesontwerpen verder uitgewerkt.

Omdat de resultaten en de onderliggende eigenschappen en fenomenen in dit proefschrift behoorlijk generiek kunnen worden geïnterpreteerd, zijn de resultaten te vertalen naar andere scheidingsprocessen. De voorgestelde benadering kan daarom worden toegepast om efficiëntere processen te ontwerpen voor de winning van polyfenolen of andere waardevolle componenten uit reststromen uit de levensmiddelenindustrie. De combinatie van het streven naar duurzaamheid en efficiëntie en een beter begrip van de onderliggende mechanismen kan leiden tot een nieuwe stap in het ontwerpen van dit soort processen. Een ontwikkeling van duurzamere processen vereist een goed begrip van het gedrag van complexe systemen, zoals de okara-matrix, maar ook van het thermodynamisch gedrag van de componenten en hun gedrag in aanwezigheid van de matrix voor, tijdens en na de verwerking in het proces.

Summary

Samenvatting

Resumen

La mejor utilización de subproductos, el uso más eficiente de los recursos, y un procesamiento más sustentable se han convertido en temas de la más alta importancia para la sociedad y la industria alimentaria. La producción de leche de soya resulta en un subproducto, llamado *Okara*, en grandes cantidades. A pesar de ser un subproducto, el okara contiene muchos nutrientes que podrían ser utilizados para el consumo humano. Por ejemplo, las isoflavonas, compuestos presentes en la soya, también se encuentran en el okara. Las isoflavonas son una subclase de los flavonoides, un grupo de los muchos polifenoles que existe, y se cree que poseen un efecto positivo en la prevención de cánceres relacionados con desordenes hormonales, enfermedades cardiovasculares, osteoporosis y obesidad. Uno de los desafíos más grandes al extraer isoflavonas a partir del okara es su baja concentración y la alta capacidad de ligar agua de la matriz biopolimérica del okara. Además, las isoflavonas comprenden muchas clases de compuestos con diferentes propiedades como por ejemplo su solubilidad. Esto convierte en un desafío la separación y purificación económicamente factible de estos compuestos con un solvente no toxico.

El objetivo de esta tesis es proveer más información para el desarrollo de un proceso de separación de polifenoles para el caso del okara. Se investigó el impacto de la presencia de una matriz semi-sólida en el proceso de separación, presentando en esta tesis las oportunidades y desafíos para la separación de isoflavonas a partir de okara. A través de este caso se investigó la aplicabilidad de una metodología de síntesis de procesos para los procesos de separación.

En el **Capítulo 2** se investigó el uso de etanol y agua como solventes relativamente no-tóxicos para la extracción de isoflavonas. Se obtuvo un alto rendimiento de extracción con concentraciones de etanol del 50% al 70%. Sin embargo, se obtuvieron diferentes óptimos en el rango de concentraciones de etanol de 0% a 100%, dependiendo del grupo de isoflavonas, dado que difieren grandemente en su hidrofobicidad. Los glucósidos tuvieron un óptimo en el rango de etanol de 50% a 60%, los malonil—glucósidos entre 30%-60% de etanol, y el rendimiento de agliconas aumentó monótonamente con la concentración de etanol. Las opciones de solvente no solo determinan el rendimiento de las isoflavonas en el extracto, sino que también el hinchamiento de la matriz, importante para la separación del solvente y el okara, y la pureza del extracto. Un primer desafío es, entonces, encontrar un buen solvente para un rango de compuestos, un segundo desafío en la utilización del okara es su alto contenido de humedad, debido a que esto limita las concentraciones de etanol que pueden ser usadas en las extracciones por solvente al extraer a partir del okara crudo. Aun así, se obtuvieron las mismas eficiencias de extracción que a partir del material seco, lo que llevó a

la conclusión que el secado inicial del material debiese omitirse, debido a que es una operación que consume una alta cantidad de energía.

Las consecuencias de usar okara húmedo (sin secado previo) se evaluaron usando análisis exergético. El uso del agua ya presente en el okara representa un desafío si una alta proporción del co-solvente etanol es necesaria, ya que aumenta la proporción líquido-sólido total. Por lo tanto, diseñamos conceptualmente el proceso de extracción y evaluamos la eficiencia y la sustentabilidad de las diferentes opciones (**Capítulo 3**). El análisis exergético puede cuantificar termodinámicamente los efectos combinados del consumo del solvente y la energía empleada. Además, puede indicar la eficiencia en el uso de los recursos de un proceso, y puede ser usado para comparar corrientes con diferentes solventes tales como etanol y agua. Aunque el análisis exergético es usado a menudo para optimización de procesos, investigamos el uso de análisis exergético dentro de síntesis de procesos, lo que entregó información más profunda para el diseño del proceso y toma de decisiones. Se determinó que un paso de secado inicial era, de hecho, menos dañino que un aumento en el uso de solventes. El uso de etanol, su pérdida y su destilación, y la pérdida del okara residual agotado se identificaron como los pasos más ineficientes dentro del proceso conceptual, y con esto, se obtuvieron guías sobre cómo mejorar la sustentabilidad del sistema.

El análisis realizado en el Capítulo 3 demostró que se lograría un paso significativo hacia una mayor sustentabilidad si el etanol se omite por completo como solvente en el paso de extracción. Además, en el Capítulo 2 se mostró que parte de las isoflavonas son más bien solubles en agua debido a su naturaleza glucosídica. Por lo tanto, la extracción y solubilización de isoflavonas en agua fue investigada en detalle en el **Capítulo 4**. Además, se investigó la co-extracción de proteínas en medio acuoso y su efecto en la extracción de isoflavonas. La temperatura no influenció la extracción, pero un aumento en la relación líquido-sólido y el pH tuvieron una clara influencia en el rendimiento de extracción. El okara puede contener también agliconas menos polares que no se encuentran naturalmente en la soya, sino que son producto de la hidrólisis de los glicósidos debido a ciertas condiciones de proceso. La ionización (disociación) de los compuestos a alto pH puede modificar su solubilidad e interacción con la matriz. Por lo tanto las agliconas también pudieron ser solubilizadas en agua mediante el ajuste del pH.

Una mayor purificación basada en separación por afinidad demostró ser factible usando agua como único solvente, dado que la mezcla agua-etanol sería tan buen solvente que la adsorción en una columna se vería dificultada. La manera normal de regenerar tales compuestos incluye el uso de un solvente diferente al usado para la extracción, resultando en un paso adicional de

evaporación. La idoneidad del agua como solvente en el paso primario de extracción quedó demostrada por la mayor afinidad de las isoflavonas por la resina en ambiente acuoso y la posibilidad de omitir un paso adicional de preparación. Los extractos resultantes son más idóneos para una posterior purificación cromatográfica, mientras que el etanol, o la mezclas agua-etanol, pueden ser utilizados como eluyente. Esto demuestra que es importante no diseñar los pasos de proceso de forma aislada, sino que optimizar el sistema de procesamiento completo de forma coherente como un todo.

El **Capítulo 5** versa sobre la adsorción de las isoflavonas en polyvinyl polypyrrolidona (PVPP) con especial atención en el efecto sobre las proteínas que se hayan co-suspendidas en el extracto. Si se toma en cuenta las isoflavonas perdidas junto a las proteínas que precipitan a bajo pH la eficiencia de adsorción fue influenciada negativamente. La afinidad de los grupos de isoflavonas pueden ser descritos con coeficientes de partición resultantes de un modelo simple. Además, la adsorción de las isoflavonas en PVPP a partir de la matriz completa de okara en su pH natural puede describirse con un modelo usando el coeficiente de partición que describe la afinidad al PVPP y a la matriz. El modelo sugiere que es posible “atraer” las isoflavonas desde el okara por adsorción concurrente de las isoflavonas a una resina de adsorción presente en la misma solución, aumentando así tanto la eficiencia como la pureza.

Los tres grupos principales de isoflavonas presentes en el okara se comportan de manera diferente a través de todo el estudio, debido a sus diferentes propiedades. El orden de sus afinidades de adsorción resultó inverso a su extractabilidad con agua. Un proceso de recuperación integrado más sustentable puede ser diseñado para compuestos polifenólicos a través de la identificación del balance correcto entre la polaridad y la hidrofobicidad del compuesto de interés, el solvente y el adsorbente.

La metodología de síntesis de proceso investigada, y el diseño de opciones que siguió a continuación al aplicar esta metodología se discute en detalle en el **Capítulo 6**. Se presenta un proceso simplificado de separación con extracción-adsorción simultánea, la que resultó de la síntesis de procesos. Los diagramas de flujo de dos casos bases que pueden ser investigados en profundidad en el futuro se discuten en mayor profundidad en el **Capítulo 7**.

Dado que las propiedades relevantes y los fenómenos subyacentes a los resultados presentados en esta tesis son de carácter genérico, pueden traducirse a otros procesos de separación. La misma estrategia puede utilizarse para desarrollar procesos de separación más sustentable para polifenoles u otros compuestos útiles a partir de otros subproductos alimenticios. Puede lograrse un nuevo paso en el diseño de procesos por medio del uso de

sustentabilidad y eficiencia como objetivos primarios dentro del diseño del proceso, en combinación con un buen entendimiento de los fenómenos subyacentes del sistema. El desarrollo de procesos sustentables requiere de un entendimiento apropiado de los sistemas complejos con los que lidiamos como la matriz por sí misma, pero también el comportamiento termodinámico de los compuestos, y su comportamiento en la matriz y durante el procesamiento.

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Lena

About the author

CURRICULUM VITAE

Lena Jankowiak was born in Berlin, Germany, on June 18th, 1982. Her education started in Berlin, where she graduated at the Droste-Hülshoff-Gymnasium in 2001. After a year in Surrey, England, working as an Au-pair, she began the studies of Food Technology at the Technical University of Berlin. In 2006, during her studies, she went to Melbourne, Australia, to take classes of the Food Science and Nutrition programme at Deakin University. In 2008, she returned once more to Melbourne, Australia, to write her Master thesis at Food Science Australia (CSIRO) with the title “Effect of high pressure and temperature on phase transition of maize starches with different amylose content”. She graduated from the Technical University of Berlin with the major in Fruit and Vegetable Technology under the supervision of Prof. Dr. Knorr, before she started her PhD in 2010 in the Food Process Engineering group at Wageningen University, The Netherlands. Under the supervision of Prof. Dr. Boom and Dr. van der Goot, she worked on the development of a separation process for high value components from the by-product okara arising at the soymilk production, which is presented in this thesis. At present, she is relocating to Santiago de Chile to proceed with her career as a Food Engineer.



PUBLICATIONS

Jankowiak, L., Jonkman, J., Rossier-Miranda, F., van der Goot, A.J., and Boom, R.M., *Exergy driven process synthesis for isoflavone recovery from okara*. Energy, **accepted**. DOI 10.1016/j.energy.2014.07.013.

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Jankowiak, L., van Avermaete, I., Boom, R., and van der Goot, A.J., *Adsorption of isoflavones on PVPP in the presence of a soy matrix*. **submitted**.

Jankowiak, L., Sevillano, D.M., Boom, R., Ottens, M., Zondervan, E., and van der Goot, A.J., *A process synthesis approach for isolation of isoflavones from okara*. **submitted**.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific courses

- Chemical Product Centric Process Design, 2010
- Advanced Food Analysis, 2010
- Sustainability Analysis in Food Production, 2011
- Food structure and Rheology, 2012
- DSTI congress: prepared for the future, Veldhoven, The Netherlands, 2010
- DSTI workshop: Chromatography Day, Amersfoort, The Netherlands, 2010
- Workshop on energy efficient separation systems, Singapore, Singapore, 2012
- Food Process Engineering Internal Symposium, Wageningen, The Netherlands, 2010
- Netherlands Process Technology Symposium (NPS11), Arnhem, The Netherlands, 2011
- 6th International Symposium on Food Rheology and Structure (ISFRS2012), Zürich, Switzerland, 2012
- 11th International Symposium on Process Systems Engineering (PSE2012), Singapore, Singapore, 2012
- Faraday discussion: Soft matter approaches to structured foods (FD158), Wageningen, The Netherlands, 2012
- 9th European Congress of Chemical Engineering (ECCE13), Den Haag, The Netherlands, 2013

General courses

- VLAG PhD week, 2010
- Project and time management, 2011
- Effective behaviour in your professional surroundings, 2013
- Scientific writing, 2012
- Career orientation, 2013

Optional activities

- Preparation of a research proposal, 2010
- PhD Trip USA, 2010
- PhD Trip Finland/Baltics, 2012
- Researchers Days ISPT, 2010-2011
- FPE weekly meetings, 2010-2014

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Cover: Okara as produced during the soymilk production (L.J./F.R.)

Lena Jankowiak, 2014