Processing of raw materials for the isolation of phytoestrogens

Report on session 2 workpackage 5

Second open plenary Phytohealth meeting

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Colophon

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Abstract

As a part of the pan-European network on improving health through dietary phytoestrogens named 'Phytohealth' a lecture session on the processing of raw materials was held at the open plenary meeting in Hersonissos at October 27th 2004. This report is an extensive summary of this session. Additional information was gathered by a patent search on the web-site of the European patent office. Scientific literature was collected using the following two databases: Current Contents and the Food Science and Technology Abstracts (FSTA). Therefor it is possible that one can find more information that even can conflict with information presented here.

Topics covered in this report are:
- sources of phyto-oestrogens, specifically isoflavones and lignans
- processing methods to obtain the two classes of compounds
- stability and shelf life of isoflavones during food processing
- impact of bioaccessibility and bioavailability of isoflavones on food processing
- implications of EU food regulation on phyto-oestrogen processing

Finally indications are given about future research directions in two areas: [1)] the processing of raw materials to obtain phyto-oestrogens and [2)] phyto-oestrogen composition and stability during food processing.

This work is carried out in a by the European Commission funded project. Any opinion given in this report does not necessarily reflect the Commission's opinion regarding that topic.

Content

1 Introduction

At the second open plenary Phytohealth meeting in Hersonissos (Crete, Greece) on 27th to 30th October 2004 a session was organised on 'Processing of raw materials' (workpackage 5 session 2). Participants and invited speakers from industry, research institutes and universities covered in their lectures:

- the interaction between analytical extraction procedures and existing or novel process technologies
- important parameters in industrial scale production of phyto-oestrogen extracts
- novel processing technologies
- legal aspects of phyto-oestrogens

Handouts of the presentations can be found in the annex. If no handouts were available a summary of the presentation is given.

In this report two classes of phyto-oestrogens are looked upon: isoflavones and lignans. A literature study on phyto-oestrogen isolation and purification processes is presented. Databases used to collect scientific publications are Current Contents (1996 to 2004) and Food Science and Technology Abstracts (1969 - 2004). The web-site of the European Patent Office (www.espacenet.com) was visited to search patents.

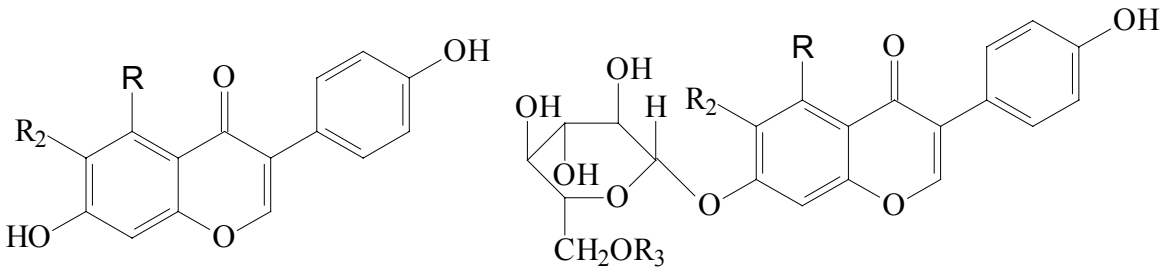
The impact of phyto-oestrogen stability during food processing on the processing of raw materials will be addressed as well as the effect of bioaccessibility and bioavailability. The implications of the EU Food regulation for applied research and innovation in the phyto-oestrogen food-processing sector will be discussed. Finally a summary of the areas of which no or little information is available is given in the conclusion chapter.

2 Isoflavones

2.1 Sources

The most well known and studied sources of isoflavones are soybeans and to a lesser extent clover. The isoflavone composition and concentration varies within a soybean. Structural formulas of the different types of isoflavones are given in figure 1. Soy endosperm or cotyledon has the highest total amount of isoflavones with traces glycitein and conjugates. In contrast the soy germ or hypocotyl contains the highest isoflavone concentration, with a relatively high amount of glycitein and conjugates. The soybean hulls contain no ro small amount of isoflavones. Dependent on the starting material (whole bean or soy germ) an isoflavone extract has a specific daidzein to genistein to glycitein ratio.

Figure 1. Structural formula of isoflavones



R1: H

R2: H daidzein
OH genistein
OCH_3 glycitein

R3: $COCH_3$ acetylglucoside
$COCH_2COOH$ malonylglucoside

After oil extraction soybean flakes can be further processed into soy protein concentrate (SPC). There are two methods to prepare SPC on industrial scale: [a)] aqueous ethanol extraction and [b)] acid wash (pH 4.5). Dependant on the process used and the isoflavone content of the raw material the isoflavon content in the final soy protein product varies.

Extraction of defatted soybean flakes with aqueous ethanol (60 – 80 v/v%) at 44 to 63 °C results in an insoluble residue, the soy protein concentrate (> 70 % protein). Isoflavones dissolve in the aqueous ethanol and the SPC is therefor low in isoflavones. To recover the ethanol the aqueous ethanol phase is subsequently evaporated. After evaporation a slurry (55 – 65 wt%) remains named soy molasses or soy soluble. This soy molasses contains among others sugars (35 wt%),

proteins (15 wt%), fat (8 wt%) and isoflavones (1 wt%). In brackets some indicative contents are given, which do vary substantially between soy molasses varieties.
In process b) defatted soybean flakes are washed with acidified water pH 4.5. The insoluble part is again SPC. Since the isoflavones do hardly dissolve in the acidified water they are therefor still present in the final product (SPC). The advantage of process b over a is that the nitrogen solubility remains high because the proteins are not denaturated like in process a.

Soy protein isolate (SPI) can be made from SPC by various methods like ultracentrifugation, sucrose density gradient centrifugation, ultrafiltration and diafiltration. The SPI has a minimal protein content of 90 %, whereas SPC has a minimal protein content of 70 %.
SPI can also be made by aqueous extraction of milled soybeans with water and hydrogenperoxide at 60 °C and pH 9. Another option is the separation of intact protein bodies by fine milling and density flotation. It is not known yet what the isoflavone behaviour is like in those two processes.

An Internet search on isoflavone extracts from clover and therefor rich in formononetin resulted in one supplier: www.ChineseHerbalExtract.com. The author is not aware of any other commercial suppliers of clover extracts for food purposes.

Daidzein and genistein content of a range of plant foods were determined by Mazur et al. and Hoeck et al. and a summary of those data presented in his thesis is given in table 1 (Mazur 2000) (Hoeck, Fehr et al. 2000). More information on the isoflavone content in foods published in scientific journals can be found in the VENUS database on the Phytohealth website (www.phytohealth.org).

Table 1. Isoflavone content of various foods, adapted from Mazur and Hoeck (Mazur 2000) (Hoeck, Fehr et al. 2000) [1].

Plant foods Trivial name (*botanical name*)	Daidzein [µg/100 gr d.w.]	Genistein [µg/100 gr d.w.]
Grains and cereals		
Rye (*Secale cereale*), whole meal	0	0
Wheat (*Triticum dicoccum*), white meal	trace	trace
Barley (*Hordeum spp*), whole meal	14.0	7.7
Oats (*Avena sativa*), white meal	0	0
Oilseeds and nuts		
Flaxseed (*Linum usitatissiumum*)	0	0
Soybean (*Glycine max*)[1]	353000	270000 – 356500
Sunflower seed (*Helianthus*)	8.0	13.9
Clover seed (*Trifolium spp*)	178.0	323.0
Walnut (*Juglans nigra*)	5.0	trace
Hazelnut (*Corylus avellana pontica*)	trace	trace
Fruits		
Apple (*Pyrus malus*)	12.4	trace
Plum (*Prunus domestica*)	0	0
Banana (*Musa sapientum*)	0	0
Papaya (*Carica papaya*)	0	0
Guava (*Psidium guajava*)	0	0
Cruciferous vegetables		
Cabbage (*Brassica oleracea*)	trace	trace
Broccoli (*Brassica oleracea italica*)	6.0	8.0
Cauliflower (*Brassica oleracea botrytis*)	5.0	9.0
Allium vegetables		
Onion (*Allium cepa*)	0	0
Garlic (*Allium sativum*)	trace	trace
Other vegetables		
Potato (*Ipomea batatas*)	0	0
Carrot (*Daucus sativus*)	trace	trace
Pepper (*Capsicum species*)	0	0

2.2 Processing methods

Currently used methods to prepare isoflavone rich extracts seem to be derived from analytical methods used to measure the isoflavone content. It is outside the scope of this report to review available analytical methods for isoflavone analysis. However for the analysis in foods often sample pre-treatment is necessary. Such a pre-treatment can be an extraction or a chromatographic purification, and those methods can sometimes be used for larger scale processing as well. There are two extensive reviews available on the analysis of isoflavones, one prepared by Hoikkala et al and one by Wilkinson et al.. Updates of those reviews are given in appendix 1 and 2 (Hoikkala, Schiavoni et al. 2003) (Wilkinson, Wahala et al. 2002).

Two types of analysis methods can be distinguished: quantitative and qualitative analysis. For quantitative analysis often isoflavones are hydrolysed to lower the detection limits. Disadvantage is the loss of information of the degree of glycosilation of the isoflavones in the matrix.
There are two ways to hydrolyse the isoflavones: using an acid treatment or by enzymatic hydrolysis using β-glucosidase (with sulfatase).
If hydrolysis is to be prevented sample extraction is carried out by a solvent extraction. Often used solvents are acetonitril / water mixtures and alcohol / water mixtures. Food samples are being extracted with aqueous alcohol (60 to 80 %) or aqueous acetonitril at temperatures between 4 and 80 °C between 1 and 19 hours. Wilkonson and co-workers mention in their article that extraction recoveries are generally at about 90 %, the more recent articles show recoveries between 90 and 115 % per compound (Wilkinson, Wahala et al. 2002).

There is some analogy between large-scale production of extracts and extraction procedures used in analysis. In both cases straightforward procedures are used, preferably with a limited number of solvents and relatively simple equipment. Dr.-Ing. Evelyn Wolfram (Frutarom Ltd.) presented in her lecture some hurdles that have to be taken into account during the scaling-up and manufacture of isoflavone extracts from soy. Frutarom Ltd.produces SoyLife®, a soy germ extract, marketed by Acatris B.V.. Batch-to-batch variation of the starting material, overall process volume, process time, ratio solid and liquid phase, procedures to adjust pH and drying methods differ substantially between laboratory scale and industrial scale. Robustness of the process is a prerequisite because the large scale overall process is less easy to control or adjust than on small scale. The question of when and how energy is to be put into increasing product yield is no longer only a strictly yield number, but economics play a major role in such a decision. A process using organic solvents can only become economically feasible if long-term recycling of the solvent is possible. During manufacturing up a fast, simple, cheap and suitable analytical method is needed to monitor extraction, like a dry matter content determination or pH or conductivity check. For quality control of the final product more laborious or expensive analytical procedures like HPLC or GC are possible, but during scale-up and manufacture it is simply too expensive.

2.2.1 Patents on the isolation or purification of isoflavones

There are quite some patents on the isolation or purification of isoflavones. Below only four different ones are given. In nearly all patents the separation is based on solubility differences of one or more isoflavones and other matrix materials like carbohydrates and proteins. The solubility property varies even between daidzein, genistein, glycitein and their different glycosides, so it is technically possible to control for example the daidzein to genistein ratio.
Of each patent source, separation principlel and a brief description of the claimed process is given.

Preparation of isoflavones from legumes, WO98/49153. (Kelly 1998)
Source: *Leguminosae* more specific soy, clover, lentils, chick peas, fenugreek and alfalfa.
Separation principle: solubility of aglycons in organic solvents (C1-C10) / extraction
Brief description
The process steps are relatively simple: first mix water + enzyme + plant material together with organic solvent, separate water and immiscible organic solvent and / or evaporation of organic solvent. Finally dry the residue rich in isoflavones (aglycones). The use of the deglycolisating enzymes ß-glucanase and ß-xylanase is claimed.

Production of isoflavon enriched fractions from soy protein extracts, US6171638. (Gugger 2000)
Source: soy whey or soy molasses
Separation principle: difference in solubility in aqueous solutions / extraction
Brief description
The outline of the patented process is as follows. Pre-concentrate the soy slurry using a membrane separation like ultrafiltration or reverse osmosis. Perform a column separation using an adsorbing resin (eg Amberlite XAD4) and elution with aqueous ethanol, evaporate the alcohol and subsequently dry the solid residue containing isoflavones. Remove undesirable color and flavor fractions by dissolving in water, separate soluble and insoluble fraction and finally dry or spraydry the insoluble fraction enriched in isoflavones.

Method for purifying and separating soy isoflavones, WO2004/043945. (Dobbins 2004)
Source: soy isoflavone concentrates
Separation principle: difference in solubility of the various glycosides in acidified organic or aqueous solutions / extraction
Brief description
In acidified solutions the solubility of genistin is lower compared to that of the other isoflavone glycosides. By reducing the polarity of such an acid solution by the addition of co-solvents such as hexane, actone or ethylacetate the solubility of daidzin and glycitin decreases. The ratio genistin-to-daidzin can be changed from 1.2:1 to 3.5:1 by refluxing soy concentrate in 16%

concentrated hydrochloric acid in methanol at 66 °C for 15 minutes and filtrating the solution using Whatman #541 filter paper (recovery 72%). Higher enrichments and recoveries can be obtained by converting the glucosides into aglycones.

Soy isoflavone concentrate process and product, US2004/0121059. (Singh 2004)
Source: soy molasses
Separation principle: difference in size and solubility of isoflavones and other compounds in water and ethylacetate / membrane filtration and extraction
Brief description
A general description of the method followed is: dilute soy molasses with water, ultrafiltrate the solubles at 60 to 82 °C using membranes having a MWCO (molecular weight cut off) between 5000 and 100.000 Dalton preferably 15.000 Dalton. The isoflavones permeate through the membrane and can be recovered from the permeate by extraction at 20 to 25 °C using ethylacetate in a ratio 5:1 (ethylacetate : permeate). Water and ethylacetate phase can then be separated using a decanter or centrifuge. Isoflavones in a solid form are collected after evaporating the ethylacetate and drying the solid residue. The residue contains over 50 wt % isoflavones.

2.2.2 Scientific literature on the isolation or purification of isoflavones

Besides the patented extraction and chromatographical methods scientific research has been focussed on two objectives:
- purification of isoflavones
- novel or modified processes for the isolation of isoflavones

In some cases both objectives could be combined. Purified isoflavones can be used as standards in analysis and to study *in vitro* and *in vivo* effects of single compounds. When there is a thorough scientific base showing a positive health effect, those processes can possibly be used by pharmaceutical industry. For food or feed purposes (bulk production, lower profit) those purified products are less interesting because of the expense of those purification processes. Food and feed industry has more benefit from novel or modified processes with which products enriched in isoflavones can be produced.

Purification of isoflavones
High speed countercurrent chromatography (HSCCC) is an all liquid chromatographic technique that combines centrifugation and separation based on hydrophobic differences. It is a non-thermal process and therefore well suited to isolate labile natural products on a preparative scale. At laboratory scale up to 30 gram sample can be applied in a single run.
(Degenhardt and Winterhalter 2001) were able to completely separate daidzin, genistin and 6”-O-acetyldaidzin by HSCCC using a mixture of tert. butyl-methyl ether/ acetonitrile / water / n-butanol as solvent system. The overall HSCCC time after injection was 8 hours. The less dense

layer was used as stationary phase and replaced by the denser layer after sample injecton. As a starting material hexane extracted toasted soy flour was used after a clean-up using an Amberlite XAD-7 column to remove proteins. For the isolation of 6"-O-malonylgenistin a different starting material had to be used: defatted soy flour without any clean-up treatment. It was observed that during the XAD-7 clean-up step malonylated isoflavones were lost, so therefor this step was omitted.
(Yang, Ma et al. 2001) used a two step HSCCC to separate up to 99 % pure daidzin, genistin and glycitin from crude soybean extract. In the first step daidzin was separated in 10 hours using a mixture of chloroform, methanol, butanol and water. The genistin and glycitin co-eluted and were separated in a second 10 hour run using a solvent system of methylbutylether / tetrahydrofuran / butanol and aqueous trifluoracetic acid.

Novel and modified processes

The general trend in the development of novel separation processes is the search for increased selectivity using environmentally friendly solvents. There are several reasons to start looking for those solvents or processes. On industrial scale organic solvent extraction is expensive because the entire system has to be explosive proof. Secondly recycling of solvent is needed and quite often distillation is used for it, implying additional heating and condensing costs. Thirdly the legally allowed amount of organic residue in the final product (extract) asks for a thorough drying step.

An example of a novel process is the MAAS technology: a continuous hybrid process combining high throughput membrane filtration and high selectivity adsorptive separation. This process was originally developed by TNO-MEP (Apeldoorn, The Netherlands) for waste water purification (Sluys and Hanemaaijer 1996). It can not directly be transferred to food industry. A range of potentially interfering components like fat, carbohydrates and proteins are present in food processes. In affinity chromatography separation is achieved by the adsorption of compounds onto affinity beads or affinity ligands. In membrane filtration large volumes can be separated based on differences in their size. In a MAAS system affinity beads or immobilised affinity ligands are mixed with the complex feed. The compounds of interest adsorb onto the large beads (50 to 500 μm) which are being retained by a membrane. The major part of the liquid feed leaves the system by permeating through the membrane. Desorption of the beads yields a concentrated product with the compounds of interest. The beads can be re-used if necessary after a regeneration treatment. Adsorptive processes are frequently used in the pharmaceutical industry (adsorptive chromatography) but it is an expensive batch process. Therefore it is hardly applied in the food industry, which is more a bulk market. In a project funded by Dutch authorities Agrotechnology and Food Innovations B.V. (Wageningen) and TNO-MEP (Apeldoorn) adapt the MAAS technology in such a way that it becomes economically interesting for food industry.

Other environmentally friendly solvents are supercritical carbon dioxide and superheated water, but they both require pressurised systems (50 to 400 bar) and therefor special equipment. The results of the studies are given below.

Carbon dioxide is the most commonly used supercritical fluid. In the supercritical phase liquid-like solvating power (which is related to the density) of a liquid is combined with gas-like transport properties (viscosity, diffusivity). It is a chemically inert, non-toxic, non-flammable food grade solvent that leaves no residues behind. Polar components have a limited solubility in supercritical CO_2. Modifiers like methanol change the polarity of the CO_2. Isoflavones are relatively polar (the glucosides are more polar than the aglycons) and addition of modifier is necessary. Rostagne and co-workers compared the extraction yields of conventional Soxhlet extraction (9 hrs, 80 – 90 °C, 80 % methanol), ultrasonification (1 hr, room temperature 22 kHz, 80 % methanol) and supercritical CO_2 extraction (50 °C, 360 bar, 93 % CO2/5 % methanol / 2% water) of hexane defatted milled soybeans (Rostagno, Araujo et al. 2002). The highest yield was obtained by ultrasonification, followed by Soxhlet extraction. The supercritical process showed a 60 to 80 % lower yield. It can be concluded that supercritical CO_2 (with or without modifier) is not interesting for the purification or isolation of isoflavones from soy.

Liquid water above 100 °C and below 374 °C under enough pressure to maintain the liquid phase is called superheated or subcritical water. Defatted soybean flakes were extracted with several solvents and some relevant data are given in the table below (Li Hsun, Ya Chuan et al. 2004).

Method, solvent	Temperature [°C]	Time [hr]	Total isoflavones* [µg/gr]	Recovery [%]
Soxhlet, 80 % MeOH	73	12	3948	100
Stirred, 80 % MeOH	73	12	3602	91.2
Stirred, 50 % MeOH	80	4	2492	63.1
Stirred, water	101	4	1565	39.6
Supercritical water 51 bar	130	2.5	3673	93.0

* total isoflavones = daidzein + glycitein + genistein + glycitin + daidzin

No detailed isoflavone data are given, so it is not sure if the ß-glucosides remain stable at the supercritical water conditions. The overall recovery indicates that superheated water extraction is a potentially suitable technology to isolate isoflavones.

By process modifications it might be possible to increase the extraction efficiency. Isoflavones are located in the vacuole of a soybean cell. A faster or more thorough cell disrupture by applying cell wall solubilising enzymes, microwaves or ultrasound waves can increase the extraction

efficiency. Ultrasound assisted extraction using regular solvents is compared to regular mix-stirring extraction of milled and freeze dried soybeans (Rostagno, Palma et al. 2003). Data were collected using the following range of conditions: (aqueous) ethanol, (aqueous) methanol, (aqueous) acetonitril at 10 °C and 60 °C. The modified process using ultrasound during extraction showed for the analysed isoflavones (daidzin, glycitin, genistin, malonylgenistin) in all cases an equal or higher extraction yield.

2.3 Stability and shelf life during food processing

The review by Faraj deals with both the influence of food processing on stability of isoflavones and health benefits of isoflavones (Faraj 2004). The currently used processing methods on industrial scale looked at are: milling and oil extraction, roasting and toasting, soy protein manufacture, fermentation, hot aqueous extraction and extrusion processing. Only some general trends will be mentioned here. There are no data showing a significant loss in or transformation of isoflavones during milling or oil extraction. The malonyl- and acetyl glucosides are susceptible to heat and conversion into the more heat stable ß-glycosides is regularly found. The thermal stability varies for glycitin, genistin and daidzin, daidzin is most stable (Xu, Wu et al. 2002). During the production of fermented products like miso and tempeh glycosides are converted into aglycones. Products made by extrusion processing (e.g. breakfast cereals, snack foods) undergo a change in isoflavone composition and concentration. Interactions with other blended and co-extruded products do have an effect too, as was mentioned already by Mahunga et al.. (Mahungu, Diaz Mercado et al. 1999).

They also describe the influence of extrusion of soy / corn blends on the amount of glycosides and aglycons (Mahungu, Diaz Mercado et al. 1999). Generally prepared blends need to be soaked with water overnight prior to extrusion to get a favourable texture of the product. The amount of water has an influence on the isoflavone profile. Water addition (up to 26 wt %) to the blend lead to a large increase in the aglycon content because ß-glucosidase is active. At the elevated temperatures during extrusion (up to 150 °C) decarboxylation of malonylglucosides into acetylglucosides occurs. It was reported earlier that de-esterification to simple glycosides can occur during hot aqueous extraction as happens during the production of tofu or soymilk (Coward 1998). No similar finding was observed by Mahunga et al. during extrusion possibly because of the much shorter residence time at elevated temperatures (Mahungu, Diaz Mercado et al. 1999). During fermentation the simple glycosides are being converted into the aglycones. This also happens when foods are burned.

A review on the stability and shelf life of isoflavones during food processing and storage was written by Shimoni (Shimoni 2004). In a growing market of functional food products and nutraceuticals the stability of the bioactive compounds is important. However there are little data available on the relationship between change in isoflavone structure and composition and its

effect on the bioactivity. Available models on isoflavone conversion and degradation appear to be simplistic, and this knowledge gap is worthwhile closing.

2.4 Impact of bioaccessibility and bioavailability on isoflavone processing

During the digestion of foods containing isoflavones the isoflavone profile (amount of malonyl-, acetyl, ß-glycoside and aglycon) changes. When studying the *in vitro* bioavailability one actually looks at the bioaccessibility (Walsh, Zhang et al. 2003). This is the degree in which isoflavonoids partition into the aqueous fraction during a digestion phase.

Generally *in vitro* studies seem to underestimate the achievable bioavailability. Nevertheless those models can be very useful in the development of food processes or phyto-oestrogen fortified food products.

It is frequently stated that the bioavailability of glycosides in humans is high compared to that of aglycons. The aglycones are less water-soluble than the glycosides but their bioaccessibility is enhanced when more bile is added during the simulated digestion. It is known that ingestion of foods containing protein and lipids induces release of bile into the duodenum (Otsuki 2000).
During soy or food processing it is important to avoid undesired conversion of isoflavones if it affects the bioavailability. As an example the conversion of glycosides into aglycons can be minimised by preventing intensive heating, low pH and activity of enzymes like β-glucosidases.

In conclusion it can be stated that both food process conditions and the food matrix can have a significant influence on the bioavailability of isoflavones. Knowledge about possible conversions or interactions is needed when one wishes to control a food process and the product quality.

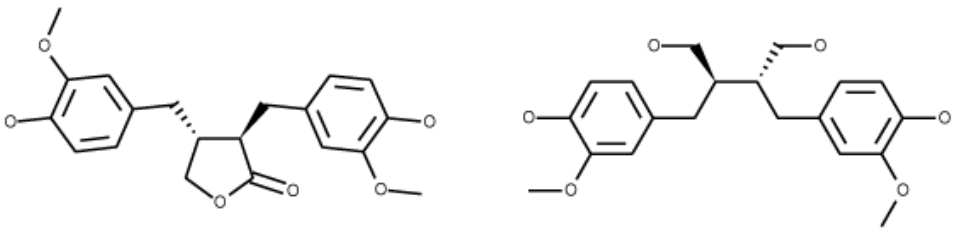
3 Lignans

3.1 Sources

The major source of lignans in the European diet comes from the oilseed flax or line seed (see table 2 on the next page). More plant foods contain lignans like secoisolariciresinol and its glycosides and matairesinol than isoflavones. It is therefor likely that the overall consumption of lignans in Europe is higher than the consumption of isoflavones. The structural formulas of these two lignans are given in figure 2. Flaxseed hulls are the best known source of the plant lignan SDG (secoisolariciresinol diglucoside). Those hulls are also rich in insoluble fibre called mucilage (4 % on dry matter basis) which has a laxative effect. Addition of 16 weigth % of flax seed decreased the weight gain in broiler chicken (Alzueta, Rodriguez et al. 2003). The antinutritional effects caused by feeding linseed were partially overcome by substituting demucilaged linseed for linseed in the diet.flax seed.
More information on the lignan content in foods published in scientific journals can be found in the VENUS database on the Phytohealth website (www.phytohealth.org).

The flaxseed husk consists of three separate anatomic parts. The outer part consists of a water-soluble mucilage, the epidermic layer. Under the mucilage, there is the actual husk substance, the spermoderm, consisting of four layers. This spermoderm is rich in fibres and lignans. The inner husk surface is the endosperm, and the seed core consists of the cotyledon, which accounts for about half of the weight of the seed. The major portion of the oil and protein of the seed is located in the cotyledon. Seeds have been used as such or in a ground form in animal feed and human nutrition.

Figure 2. Structural formula of matairesinol (left) and secoisolariciresinol (right).



3.2 Processing methods

Because the flax hulls stick to the embryo separation is difficult even after defatting. Dave Oomah reported in 1998 on a suitable method to dehull flaxseed by abrasive dehulling only and by a combination of microwave drying and abrasive dehulling (Dave Oomah and Mazza 1998). They did not look at the effect of processing on the SDG content.

Wiesenborn and co-workers were able to double the SDG content on dry matter basis by separating the hulls from defatted flaxseeds (Wiesenborn 2000). The overall yield of this milling in a Comitrol® processor, sieving and air classification process was low compared to similar reported processes. The SDG content appears to be inversely correlated with the oil content. As an alternative a barley pearler followed by sieving and a gravity table was tested. Both hull and oil yields were higher, whereas the SDG concentration was less increased compared to the first process.

The patent of Myllymaki describes the manufacture of lignan rich powder from (defatted) flaxseed by abrasion in a mill equipped with millstones or in a modified rice polishing device (Myllymaki 2000). An additional bleaching of the powder with a mixture of alcohol, water and hydrogenperoxide deflavours and/or degreases the fibrous material. Data show that the longer the abrasion time (1 to 8 minutes) the more fat is present in the fibrous product (18 to 36 wt%). Similar to the finding of (Wiesenborn 2000) the lignan concentration is inversely related to the fat concentration. After 1-minute abrasion the lignan contents was 1480 mg/100gr and this went down to 800-mg/100 gr when the abrasion time increased to 8 minutes.

Flaxseed can be separated on industrial scale in oil (by pressing or liquid CO_2 extraction), an lignan-enriched fraction (the hulls) and a protein rich fraction (the residue). For further lignan concentration extraction procedures are needed.

3.2.1 Patents on the isolation or purification of lignans

Similar to the isolation or purifcation of isoflavones lignan concentration can be achieved by solvent extraction possibly in combination with a chromatographic purification. Two patents are given below showing the possibilities. Compared to isoflavone extraction the flaxseed matrix contains more interfering compounds. To minimise off-flavours in the final product more extensive treatment is needed. Also the stability of purified and dried lignans is less compared to isoflavones because they are very hygroscopic. Besides the difficulties in handling and formulation the moist supports the growth of molds and bacteria. The second patent gives a stabilisation procedure: a mixture of oil and if necessary addition of exogenous anti-oxidants like tocopherols or carotenoids.

Table 2. Lignan content of various foods analysed using ID-GC-MS-SIM, adapted from (Mazur 2000).

Plant foods Trivial name (*botanical name*)	Total secoisolariciresinol [µg/100 gr d.w.]	Matairesinol [µg/100 gr d.w.]
Grains and cereals		
Rye (*Secale cereale*), whole meal	47.1	65.0
Wheat (*Triticum dicoccum*), white meal	8.1	0
Barley (*Hordeum spp*), whole meal	58.0	0
Oats (*Avena sativa*), white meal	13.4	trace
Oilseeds and nuts		
Flaxseed (*Linum usitatissiumum*)	369900	1087.1
Sunflower seed (*Helianthus*)	610	0
Clover seed (*Trifolium spp*)	13.2	trace
Walnut (*Juglans nigra*)	163.0	5.0
Hazelnut (*Corylus avellana pontica*)	119.0	4.0
Fruits		
Apple (*Pyrus malus*)	trace	0
Plum (*Prunus domestica*)	5.0	0
Banana (*Musa sapientum*)	10.0	0
Papaya (*Carica papaya*)	8.2	0
Guava (*Psidium guajava*)	699.7	trace
Cruciferous vegetables		
Cabbage (*Brassica oleracea*)	33.0	trace
Broccoli (*Brassica oleracea italica*)	414.0	23.0
Cauliflower (*Brassica oleracea botrytis*)	97.0	trace
Allium vegetables		
Onion (*Allium cepa*)	83.0	8.0
Garlic (*Allium sativum*)	379.0	3.6
Other vegetables		
Potato (*Ipomea batatas*)	10.0	6.0
Carrot (*Daucus sativus*)	192.0	3.0
Pepper (*Capsicum species*)	117.0	7.0
Beverages, wines (origin)		
Chardonnay (Italy), white	135.5	17.2
Cabernet Sauvignon (France), red	686.0	74.1

Process for extracting lignans from flaxseed, US5705618. (Westcott 1998)

Source: extracted and defatted flaxseed

Separation principle: liquid / liquid separation (extraction) followed by reversed phase chromatography

Brief description

Oil-free flaxseed meal was first extracted for 24 hours with a mixture of 70 % ethanol / 30 % water in a liquid: solids ration of 6:1. After separation of solid and liquid by filtration the extract was evaporated and further dried. Then a base-catalyzed hydrolysis of the lignans using triethylamine in anhydrous methanol was performed.

The hydrolysate was subjected to liquid / liquid partitioning using ethylacetate / water in the ratio 4:1. The SDG dissolved in the aqueous phase and was purified further by chromatography after removal of residual ethylactetate by vacuum evaporation. The chromatographic system can consist of either a reverse phase C18 resin or an anion exchange resin. By reverse phase impurities were eluted using 1 % acetic acid. SDG enriched fractions were collected by elution with 1 % acetic acid in 30 % methanol. The vacuum evaporated product contained SDG at a purity of 60 % on weight basis.

As anion exchange resin A-25 QAE Sephadex can be used prepared in the acetate counterion form. SDG was eluted with water after application of the pH 5 adjusted hydrolysate. After a solvent switch to 50 % acetic acid in 15 % ethanol acid components including glycosides of cinnamic acids were eluted.

Further purification (up to > 90 %) was achieved by preparative scale HPLC suing a C18 reverse phase column applying a gradient of 100 % aqueous acetic acid to 40 % aqueous acetic acid / 60 % methanol.

Process for extraction and stabilization of phyto-oestrogens from flaxseed and product therefrom, US2003060420. (Benett 2003)

Source: oil free flaxseed meal or flaxseed hulls.

Separation principle: difference in solubility in aqueous solutions / extraction.

Brief description

After mixing the source and 50 to 85 % aqueous ethanol for 1 to 6 hours at 10 to 60 °C, solid and liquid phase are separated using a 25 to 250 μm filter. Ethanol is recovered from the coloured liquid phase by vacuum evaporation (50 to 70 °, 500 to 600 mmHg). The remaining aqueous lignan fraction can be hydrolyzed at pH 12 to 13, for 1 to 2 hrs at 40 t 70 °C, to generate uncomplexed lignans. After hydrolysis the pH of the mixture is decreased to pH 7 to 8 by adding hydrochloric acid. The material is freeze dried and finally mixed with a vegetable oil to stabilise the hygroscopic lignans. Together with some other compounds the lignan secoisolariciresinol diglucoside (SDG) is extracted following the before mentioned procedure.

3.2.2 Scientific literature on the isolation or purification of lignans

Little literature is available on isolation or purification procedures for lignans from flaxseed other than the patents mentioned before. If the scope is expanded to lignans from sesame, then a few other come up although they mainly focus on analytical procedures. Additional information on flaxseed in human nutrition can be found in the book of Cunnane (Cunnane and Thompson 1995). In four book sections the following topics are being addressed: [a)] composition and agronomic aspects, [b)] metabolic effects, [c)] clinical applications and [d)] human food products. Although it is published in the US 10 years ago, it can be a good starting point when one is interested in flaxseed production and utilisation.

Similar to their isoflavones purification SDG was purified by high-speed countercurrent chromatography (HSCCC) using as solvent system a mixture of tert. butyl-methyl ether/ acetonitrile / water / n-butanol (Degenhardt, Habben et al. 2002). The SDG was isolated from flaxseed after grinding, defatting, hydrolysis with 1 M sodiumhydroxide, XAD-2 chromatography and HSCCC. The purity of the obtained SDG was determined by HPLC and appeared to be over 93% based on peak area. The large number of process steps already show that this technology is not directly applicable on large scale. The procedure is useful when purified SDG is needed for research purposes like for studying the *in vitro* or *in vivo* bioavailability.

4 Implications of EU food regulation on phyto-oestrogen processing

In this chapter deals with the impact of EU food regulation on phyto-oestrogen processing. To outline some future perspectives of isoflavones and lignans, the history and current status of the by EU approved phytosterols is looked upon.

Next to isoflavones and lignans there are numerous plant compounds which can bind to the mammalian estrogen receptor. Fritsche et al. wrote a review on the occurrence of hormonally active compounds in food (Fritsche and Steinhart 1999). They mention the following classes:
- isoflavones (daidzein, genistein, glycitein, biochanin A, formononetin)
- coumestans (coumestrol)
- lignans (matairesinol, secoisolariciresinol)
- phytosterols (β-sitosterol, campesterol, stigmasterol and dihydrobrassicasterol)
- other flavonoids (chalcones, flavones, flavonol, flavanones).

In brackets the most known compounds of that class are given. Although phytosterols have been consumed for ages the fortified products, like the margarine 'Becel pro-active', are regarded as novel foods. After the EU approval of this product more companies apply for the approval of phytosterol enriched products. For example Coca-Cola Services applied for the approval of juices and nectars with added phytosterols in October 2004. The phytosterols used in this last case are derived from tall oil soap (Cargill, trademark CoroWise, up to 88 wt % phytosterols or phytosterol esters).

Phytosterols or plant sterols are present in cereals like rye and barley and they are already being consumed in bakery products. In plants they occur as 4 types of conjugates: esterified to a fatty acid or a hydroxycinnamic acid or glycosylated with a glucose or a 6-fatyy-acylhexose (Moreau, Whitaker et al. 2002). Phytosterols are not completely saturated molecules whereas phytostanols, which are a subgroup of phytosterols, are fully saturated. Clinical studies on the effect of phytosterols or phytostanols tend to be uniform in their results: consumption of 1 to 3 gram phytosterols or phytostanol a day lowers the levels of total and LDL cholesterol in plasma (Moreau, Whitaker et al. 2002). Miettinen and co-workers recently reported that after one year consumption of a spread fortified with either phytosterols or phytostanols, the phytostanols have a significantly larger effect on the cholesterol lowering effect (Miettinen and Gylling 2004). As reported by Clifton there is a food matrix effect on the cholesterol lowering effect of plant sterol esters (Clifton, Noakes et al. 2004). The effect was significantly larger when plant sterol esters were formulated into milk compared to bread and cereals.

Isoflavones and flavonoids are studied classes in terms of occurrence in plants, *in vitro* and *in vivo* bioavailability, and conversion during digestion and (beneficial) health effects. Like phytosterols they occur in plants as glycosides and in the long track of production, processing and digestion they are converted into metabolites that can have an effect on cell level. Commercially available

isoflavone rich extracts are soy based materials, containing up to 70 wt % isoflavones. Some soy isoflavone brands are SoyLife ™ (Acatris B.V.), Solgen ™ (Solbar Ltd.), AdvantaSoy™ (Cargill Inc.), FujiFlavone™ (Fujicco) and NovaSoy™ (ADM).

The EU regulations regarding foods can be divided in five main areas:
- basic regulation
- food safety
- consumer information
- novel food / GM food
- food supplements

Both the basic regulation and food safety are covered by EC regulation no. 178/2002 of the European Parliament and of the council. The main principles behind this regulation are safety for consumers, the precautionary principle, risk analysis and information mechanisms.

Consumer information is covered by Directive 2001/95/EC of December 2001. It basically regulates that authorities can request all necessary information from all concerned parties and that there is a general obligation to interchange information especially in the case of risks. Directive 2000/13/EU deals with the labelling of food products. It also mentions a list of most important allergenic ingredients that should be mentioned on the label. There is a final draft version on the 'Regulation on nutrition and health claims made on foods' COM(2003) 424 which was adopted by the EC on 16th July 2003. It distinguishes three types of claims: nutrition claim, health claim and a reduction of disease risk claim. Once a claim is authorised it can be used without any new authorisation. Health claims need explicit authorisation and are not allowed for 'problematic' products like potato chips. Risk reduction claims will not be forbidden anymore. Nevertheless claims are only allowed on the basis of scientific evidence for a positive physiological effect of typical quantities. Currently the possible nutrition claims is limited to a list of 24 claims mainly focussing on low or high levels of fat, sugars, salt, protein, fibres, vitamins and minerals. Internationally there are three other health claim regulations: FOSHU licensing system (Japan), FDA (USA) and the Codex Alimentarius (international). The draft EC nutrition and health regulation interferes with some already existing regulations and it tends to be stricter compared to the other international regulations.

Foods and food ingredients consisting of or isolated from plants fall within Regulation EC No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients Article 1(2). It defines novel foods as – foods and food ingredients, which have not hitherto been used for human consumption to a significant degree within the Community (before 15th May 1997). Like phytosterols isoflavone or lignan extracts could be regarded as a novel food. Only when the isoflavone extracts are regarded as soy extracts containing soy protein it might be possible that those extracts can be regarded as regular foods.

Plant extracts will not be regarded as food supplements because this category is limited to vitamins and minerals only which are listed in annex I of the Directive 2002/46/EC of 10 June 2002.

If products with phyto-oestrogens can be regarded as healthy foods this can be an opportunity for consumers to buy those products. To obtain a health claim the positive effects of the product have to be scientifically established. Besides demonstration of the quality and safety of the product also minimal and maximum intake levels have to be pointed out. Dependant on the positioning of the final product a novel food status might be necessary, similar to phytosterol containing foods.
On the following web-site the above mentioned information and more can be found on EC regulations regarding foods: http://europa.eu.int/comm/food/food/index_en.htm.

5 Conclusions

There are quite some methods available for isolation or purification of isoflavones. Research could focus on the development of environmentally friendly processes. However the overall impact of such a sustainable process is expected to be limited. This because the market of purified isoflavones seems to be rather limited unless a substantial health effect can be shown. It is worthwhile to investigate the stability of isoflavone-fortified foods and isoflavone - food matrix interactions. So far only few publications have addressed these issues.
Industrial implementation of purification of isoflavones is hampered for two main reasons:

- there is not enough scientific data for a health claim on one or more specific isoflavones
- from a legal point of view purified isoflavones are not being consumed as such and therefor isoflavone fortified foods can be considered as novel foods.

Those two reasons make that is is not profitable to implement more extensive and expensive processes.

Regarding lignans and coumestans there is even less information available on:

- sources
- effect of raw material processing
- effect of food processing
- food-matrix interactions
- stability of isolated or purified compounds

Regarding lignans possibly sources and processing are already being researched in the two year EU CRAFT project (QLK1-2002-71714) 'Developing lignan enriched functional food from linseed (*Linum usitatissimum* L.)' which started at 1st March 2003.

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Annex

Annex 1. Update 'Analysis of phyto-oestrogens in biological matrices'.

The published manuscript of (Hoikkala, Schiavoni et al. 2003) contains 95 references. Publications after 2000 are mentioned below.

HPLC analysis

Matrix	Analyte*	Pretreatment	Recovery	Internal standard	Column	Mobile phase	Detector	Detection limit	Reference
Urine	D, G, GL Free isoflavones (Fr), total isoflavones (T), free plus sulfate conjugates (FrS)	Centrifugation, extraction MBE, enzymatic hydrolysis (T), C_{18}-SPE purification, acid hydrolysis, extraction (FrS)	D 94 – 102 % G 88 – 96 % GI 91 – 102 %	4-HBPH	Phenyl (75 mm * 4.6 mm, 3.5 µm)	Ammonium-formate, MeOH, gradient	UV 259 nm	D 22.3 ng/ml G 22.7 ng/ml GI 22.5 ng/ml All LOQ	(Thomas, Zeisel et al. 2001)
Plasma	D, G, GL Free isoflavones (Fr), total isoflavones (T), free plus sulfate conjugates (FrS)	See above. For FrS: lyophylisation, acid hydrolysis, extraction MBE	D 45 – 59 % G 37 – 52 % GI 34 – 47 %	4-HBPH	Phenyl (150 mm * 4.6 mm, 5 µm)	Ammonium-formate, MeOH, ACN, gradient	UV 259 nm	D 1.7 ng/ml G 2.1 ng/ml GI 2.5 ng/ml All LOQ	(Thomas, Zeisel et al. 2001)
Breast tissue Urine Serum	D, G, EQ	Homogenisation, enzymatric purification, centrifugation, enzymatic hydrolysis, C_{18}-SPE	70 ± 5.6 % breast tissue homogenate 100 % ± 14.1 % urine serum	4-HBPH	C18 (250 mm * 4.6 mm, 5 µm)	ACN- methanol, formic acid in water, isocratic	UV, 249, 230, 261 nm	Breast tissue homogenate 0.9 / 0.9 /1.9 nmol/l Urine 4.7 / 4.7 / 9.4 nmol/l Serum 18.9 / 18.9 / 37.5 nmol/l	(Maubach, Bracke et al. 2003)
Serum	D, G, GL, EQ, *O*DMA, END, ENL	Enzymatric hydrolysis, C_{18}-SPE	92 – 101 %	Deuterated standards of correspondin g analytes	C18 (150 mm * 2.1 mm, 3.5 µm)	Ammoniumacetate, MeOH, gradient	ESI-MS	10 pg/ml except EQ 100 pg/ml	(Grace, Taylor et al. 2003)
Urine	D, G, Din, Gin, F, BA	Dilution pH 5.5 acetate buffer	94 – 107 %	Standard addition	C18 (150 mm * 2.1 mm, 3.5 µm)	ACN- acetate buffer, gradient	ED	Din 480 / Gin 394 pg/l	(Klejdus, Vacek et al. 2004)
plasma	D, G	Acid hydrolysis, centrifugation, Alumina SPE	D 77 G 79, IS 86 %	4-HBPH	C8 (150 mm * 2.0 mm, 5 µm)	Ammonium-formate, ACN, isccratic	ESI-MS-MS	2.5 / 8.5 ng/ml	(Yang, Zhou et al. 2004)

4-HBPH = 4-hydroxybenzophenone, MBE= methyl tert. butylether

GC analysis

Matrix	Analyte*	Pretreatment	Recovery	Internal standard	Column	Detector	Detection limit	Reference
urine	D, EQ	SPE + HPLC	94.6 %, 97.0 %		Correlated to GC-MS			(Venturelli, Rinaldi et al. 2002)
urine	D, G, GL, EQ, *O*-DMA, END, ENL	Enzymatric hydrolysis, C_{18}-SPE	90 – 104 %	Deuterated standards of corresponding analytes	15 m * 0.25 mm, 0.25 µm, 100 % dimethylpolysiloxane	MS	END 1.2 ng/ml ENL 5.3 ng/ml	(Grace, Taylor et al. 2003)

Immunoassays

Matrix	Analyte*	Pretreatment	Recovery	Internal standard		System	Detection limit	Reference
Urine	D, G, EQ	G: none D + EQ: enzyme hydrolysis			Correlated to GC-MS and polyclonal commercial labmaster assay	TR-FIA	D 1 ng/ml G 24 ng/ml EQ 5.7 ng/ml	Isoheart project
Serum	D, G, EQ	D: enzyme hydrolysis For G + EQ followed by 2* diethylether extraction			Correlated to GC-MS and polyclonal commercial labmaster assay	TR-FIA	D 1 ng/ml G 2.3 ng/ml EQ 0.57 ng/ml	Isoheart project

Annex 2. Update review 'Identification and quantification of polyphenol phyto-oestrogens in foods and human biological fluids'.

The article of (Wilkinson, Wahala et al. 2002) contains 95 references up to the beginning of 2001. Because of the overlap between this review and the (Hoikkala, Schiavoni et al. 2003) article, in this section only attention will be paid to publications after 2000 dealing with the analysis of phyto-oestrogens in foods.

Foods

Matrix	Analyte*	Pretreatment	Recovery	Internal standard	Column	Mobile phase	System	Detection limit	Reference
Soy samples	D, G, Gl, Din, Gin, Glin, Adin, Mgin, Agin, Mglin, Aglin	Sonication and extraction80 % EtOH, 60 °C, 1 hr, centrifugation, hexane lipid removal	n.d.	$^{2}H_4$ D	C18 (250 mm * 3.2 mm) 5 µm	Acetic acid in water, ACN, gradient	LC-MS	0.1 mg/kg	(Wiseman, Casey et al. 2002)
Soybeans	D, G, Gl, Din, Gin, Glin, Mdin, Mgin, Mglin, Adin, Agin, Aglin	Qualitative: 80 % MeOH, 4 °C, overnight Quantitative: Acid hydrolysis in 64 % EtOH, 2 hr	n.d.	Formonetin	ODS (150 mm * 3.2 mm) 5 µm	Formic acid in water, ACN, gradient	LC-UV	LOQ 0.4 mg/kg	(Wu, Wang et al. 2004)
Soybeans	D, G, Gl, Din, Gin, Glin, Mdin, Mgin, Mglin, Adin, Agin, Aglin	Qualitative: 80 % MeOH, 4 °C, overnight Quantitative: Acid hydrolysis in 64 % EtOH, 2 hr	n.d.	Formonetin	ODS (150 mm * 3.2 mm) 5 µm	Formic acid in water, ACN, gradient	LC-ESI-MS	LOQ 0.1 mg/kg	(Wu, Wang et al. 2004)
Soybean seeds	D, G, Gl, Din, Gin, Glin, Mdin, Mgin, Mglin, Adin, Agin, Aglin	Formic acid pH 2 hydrolyis in 70 % EtOH overnight, hexane defatting	95 %	Formonetin	C18 (150 mm * 3.9 mm, 4 µm)	Phosphoric or formic (MS) acid in water, ACN, gradient	LC-DAD LC-MS	0.2 mg/kg	(Heimler, Vignolini et al. 2004)

Matrix	Analyte*	Pretreatment	Recovery	Internal standard	Column	Mobile phase	System	Detection limit	Reference
Bovine milk	D,G,EQ,F, BA, END,ENL, C, P	Aqueous acetone extraction, enzymatic hydrolysis, centrifugation, SiOH SPE	30 – 40 %	Prunetin	C18 (50 mm * 2.1 mm, 5 µm)	Acetic acid in water, MeOH, gradient	LC-ESI-MS-MS	1 µg/L	(Antignac, Cariou et al. 2003)
Food (with and without soy)	D, G, Din, Gin	HCl / MeOH hydrolysis 100 °C, 1.5 hr + hexane lipid removal + in tube SPME	97% - 115 %	ß-naphtol	C8 (150 mm * 4.6 mm) 5 µm	MeOH/ water, gradient	LC-DAD	0.4 – 0.5 ng/ml (=0.8- - 1.0 µg/kg!)	(Mitani, Narimatsu et al. 2003)
herb *Genista tinctoria L.*	Ain, Agin, BA, C, D, EQ, F, G, HF, Fin, GDG, GHG, Isol, L, Li, Lin, Mgin, O, Pu, Si	80 % MeOH ultrasound 4 hr, 20 °C	89 – 95 %	Isoliquiritigenin (Isol)	C18 (250 mm * 4.0 mm) 5 µm	Acetic acid in water, ACN, gradient	LC-DAD LC-ESI-MS	n.d.	(Luczkiewcz, Glod et al. 2004)
Red clover	BA, Ca, D, F, G, Gl, Iri, P, Pr, Ps	Quantitative: acid hydrolysis Qualitative: 80 % MeOH, sonication, 1 hr, 20 °C	n.d.	Not mentioned	ODS (150 mm * 3.2 mm) 5 µm	Formic acid in water, ACN, gradient	Simultaneous LC-UV and LC-ESI-MS-MS	UV 25 ng/ml MS 6 ng/ml	(Wu, Wang et al. 2003)
Soy extract Soy based health supplements	D, Din, G, Gin, Gl, Glin	Step 1. Hydrolysis 80 % ethanol / 1 M HCl, 80 °C, 1 hr. Step 2 80 % EtOH. Combine 1 and 2.	76- 119 % per compound, 100 % total isoflavones	None, external standards of analytes	ODS (150 mm * 3 mm) 3 µm	Sodium acetate buffer / MeOH / ACN, gradient	LC-CEAD	n.d.	(Penalvo, Nurmi et al. 2004) (Penalvo, Heinonen et al. 2004)
Soy extract	D, Din, G, Gin, Gl, Glin	DMSO / MeOH extraction + sonication	97 – 103%	None, external standards of analytes	C18 (100 mm * 4.6 mm)	Acetic acid in water, ACN, gradient	LC-DAD	n.d.	(Apers, Naessens et al. 2004)

Matrix	Analyte*	Pretreatment	Recovery	Internal standard	Column	Mobile phase	System	Detection limit	Reference
Soy based health supplements	Ila, La, Pi, Sy	Enzymatic hydrolysis, ether extraction, acid hydrolysis, diethyl ether: ethyl acetate extraction, chromatograhic purification, derivatisation QSM	97 - 105 %[1]	$^{2}H_{6}$-matairesinol	BP-1, 12 m * 0.22 mm, 0.25 µm		GC-MS	20-30 µg/kg[1]	(Penalvo, Heinonen et al. 2004) [1] (Mazur, Fotsis et al. 1996)
Soybean seeds	D, G, Gl, Din, Gin, Glin, Mdin, Mgin, Mglin	Extraction 80 % MeOH, 80 °C 4 hr, evaporation	n.d.	None	Fused silica, 50 µm inside, 67 cm	150 mM borate buffer pH 10.5	CE-DAD	n.d.	(Aussenac, Lacombe et al. 1998)

Abbreviations used*:

Adin = acetyldaidzin, Agin = acetylgenistin, Aglin = acetylglycitin, Ain = apigenin-7-O-glucoside, BA = biochanin A, C = coumestrol, Ca = calycosin, D = daidzein, Din = daidzin, END = enterodiol, ENL = enterolactone, EQ = equol, F = formononetin, G = genistein, Gin= genistin, Gl= glycitein, Glin = glycitin, GDG = genistin-7O-diglucoside, GHG = 2'- hydroxygenistein-7-O-glucoside, HF = 3', 4', 7-trihydroxyflavone, Ila = isolariciresinol, Iri = irilone, Isol = isoliquiritigenin, L= luteolin, La = lariciresionl, Li = liquiritigenin, Lin = luteolin-7-O-glucoside, Mdin = malonyl daidzin, Mgin = malonyl genistin, Mglin = malonylglycitin, O = ononin, P = prunetin, Pi = pinoresinol, Ps = pseudobaptigenin, Pr = pratensein, Pu = puerarin, Sy = syringaresinol

n.d. = no data mentioned in the article

Annex 3.
Summary lectures of the session 'Processing raw materials for phytoestrogen extraction'.

Of each lecture either the presented slides or a summary of the lecture is given on the following pages.

Dr.-Ing. Evelyn Wolfram (FRUTAROM Switzerland Ltd, Wädenswil).
Scale-up of extraction processes for an active multicomponent extract of linseed hulls and soy germs.

Dr. Bernhard Bührlen (Fraunhofer Institute, Karlsruhe, Germany).
Legislative aspects of functional ingredients like phytoestrogens.

Ing. Joyce Schroot (Agrotechnology and Food Innovations B.V., Wageningen, The Netherlands).
MAAS and the isolation of minor components like phytoestrogens.

Prof. Peter Winterhalter (Institute of Food Chemistry, Technical University of Braunschweig, Germany).
"Phytoestrogen" is more than soy-derived isoflavones: Additional sources, structures, and isolation methods.

R. Grougnet (University of Athens, Greece).
Isolation of new lignans from sesame agricultural waste using resin adsorption technology.

A. Agalis (University of Athens, Greece)
Isolation of natural antioxidants from olea europea leaves, fruit and waste waters.

M. Farré (Environmental Chemistry Department IIQAB-CSIC, Barcelona, Spain).
Review of the extraction procedures for analytical methods used in the determination of phytoestrogens in food and environmental samples.

Scale-up of extraction processes for an active multicomponent extract of linseed hulls and soy germs.

Wolfram, E.[1]; Verburggen, M.[2]; Bolardt, M.[1]; Peter, S.[1]; Jorns, R.[1]; Vautravers, S.[1]; Kreuter, M.H.

[1] FRUTAROM SWITZERLAND LTD., Rütiwisstrasse, CH-8820 Wädenswil, Switzerland
[2] Acatris B.V., Burgstraat 12, PO Box 1, 4283 ZAG Giessen, The Netherlands

Linseeds and soy germs are well known sources of phytoestrogens. In order to use them as nutraceuticals, the active ingredients need to be transformed into a form, convenient to used for the consumers.

The extract development was supposed to yield a high content of the leading active ingredients – either one specific substance (e.g. SDG for Linseed) or a sum of a group of leading substances (e.g. isoflavones) and had to consider the requirements concerning the later application in food or dietary supplements such as its form and suitability for further processing.

In general, the final goal of a development project from laboratory scale to scale-up into production is a robust and valid production process which lead to a standardised quality of the multicomponent system: the standardised extract as an active ingredient.

At Frutarom Switzerland, the pathway of such a development of a new extract starts in the process development department, where the first process scheme is identified on the laboratory bench, optimising yield an d content of the leading substance(s). Therefore, the laboratory equipment has to image, if possible, the equipment in large scale. Reliable accompanying analytics is essential. From these factorial experiments, critical parameters are identified and the suitable and necessary in-process controls are chosen. The most critical parameter for phyto extracts is the variability of the raw material, which has to be encountered by purchasing from known sources, a sound raw material specification and a smart process development considering this issue. A first specification with wide acceptance criteria is established. The process is ready for scale-up.

Considering the successful contract development co-operation for Linseed and Soy extract as an example, the challenges of scaling-up standardised extraction processes for active ingredients from plant materials in the pharmaceutical an d health food industry will be illustrated and a path for solving them depicted.

Legislative aspects of functional ingredients like phytoestrogens

Dr. Bernhard Buehrlen

Fraunhofer Institute Systems and Innovation Research ISI, Karlsruhe, Germany

PHYTOHEALTH 2nd Open Plenary Meeting

October 27–30th, 2004

Hersonissos-Heraklion, Crete, Greece

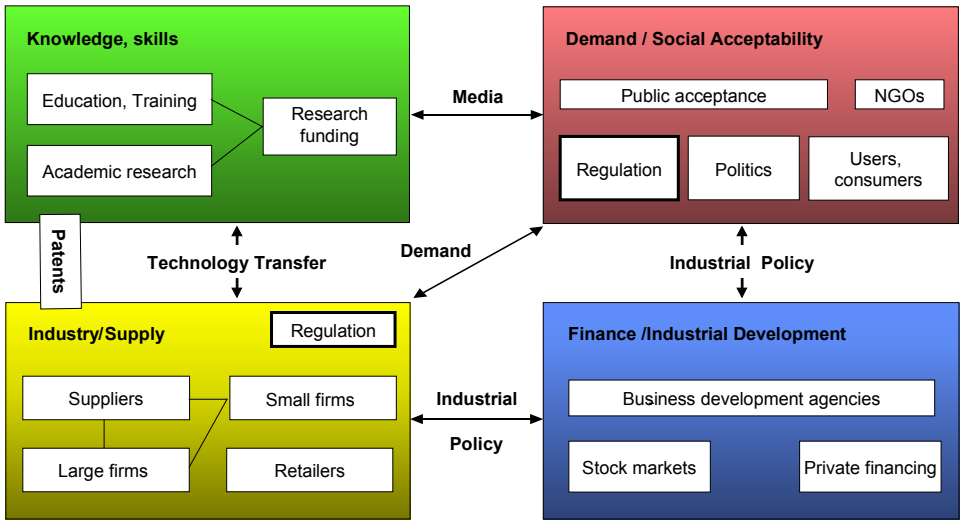
phytohealth

Fraunhofer Institute Systems and Innovation Research

Overview

- Regulation and innovation: The innovation system
- General aspects of regulation (in the food sector)
- Regulation of the food sector
  - Basic regulation
  - Food safety
  - Consumer Information
  - Novel food/GM food
  - Food supplements
- Conclusions for products with phytoestrogens

The innovation system



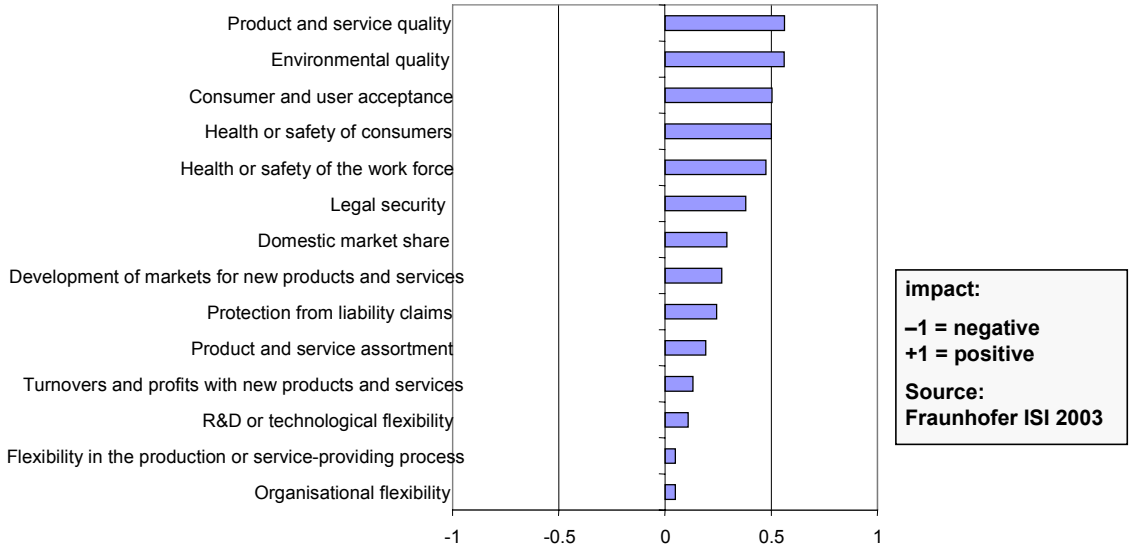
Source: Senker et al. 2001

General aspects: Targets of regulation

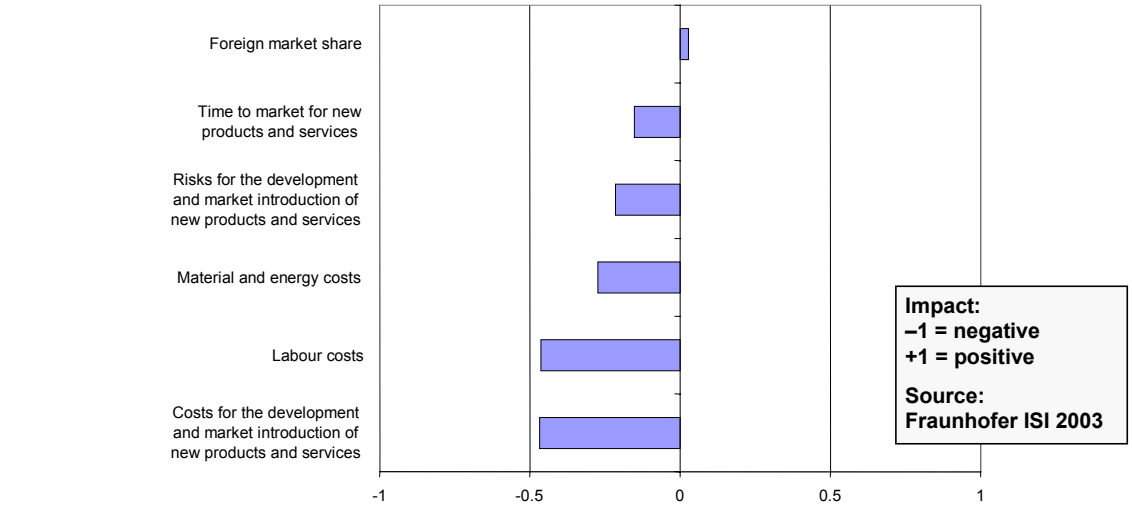
- Quality of products and services
- Competition/ Free circulation of products and services
- Health aspects
- Safety aspects
- Environmental aspects
- Prices of products and service
- Securing influence of governmental institutions

Compared to other industry sectors, food is a highly regulated matter.

General aspects: Positive impacts of the regulatory framework relevant for the introduction of new products and services



General aspects: Negative impacts of the regulatory framework relevant for the introduction of new products and services

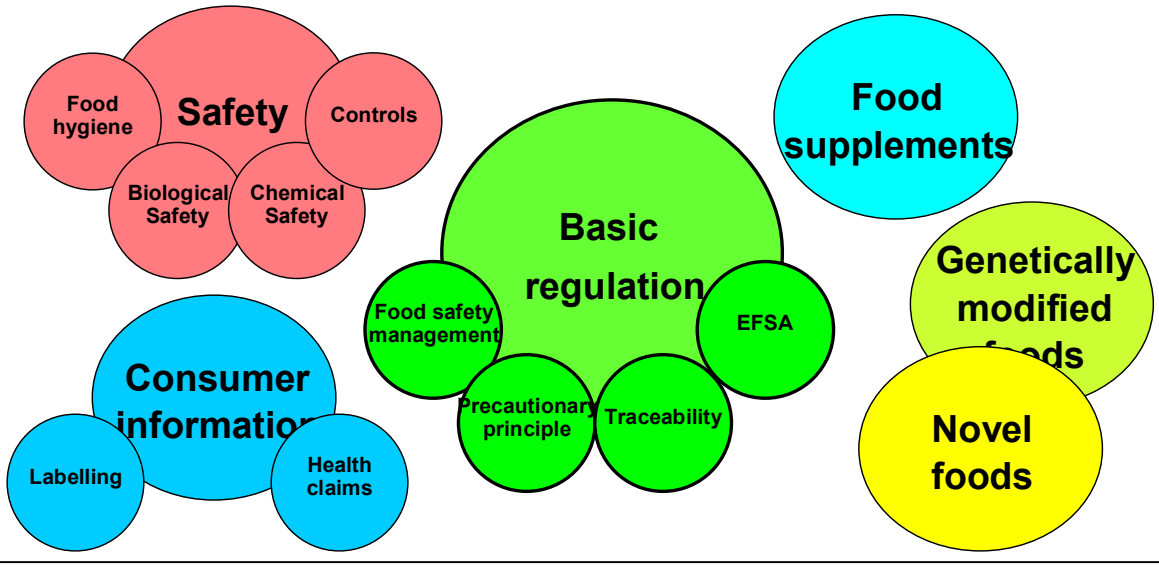


General aspects: Impact of regulation in the food industry

- Regulation gives opportunity to introduce new products and services (e.g. health claims).
- Safety of products is common concern of industry and consumers.
- Consumers and ecologists tend to more rigid regulation.
- Industry wishes simplification of approval procedures.
- Legal insecurity is a hindering phenomenon for introducing new products in the food sector (food products based on GMOs, functional foods).
- The regulatory system in the USA is more liberal regarding the marketing of GMOs and the use of health claims, but also has stronger liability claims.
- Other factors play an even more important role, especially market, attitudes of consumers, product liability, research funding...

Source:
Fraunhofer ISI 2003

Regulation of the food sector in the EU



Regulation of the food sector: Basic regulation (1)

REGULATION (EC) No 178/2002 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 28 January 2002
laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

Parts directly in force since 28. Feb. 2002, parts have to be implemented in national legislation until 2007

- Definitions
  - Foods, beverages, ”deliberate” ingredients thereof
  - Excluded: feed, pharmaceuticals, cosmeticals , tobacco (and products)
- Principles
  - Risk analysis
  - Precautionary principle
  - Safety for consumers
  - Consultation, information mechanisms

Regulation of the food sector: Basic regulation (2)

- Definitions, responsibilities
- Food safety provisions
- Import/export (requirements, responsibilities)
- European Food Safety Authority (EFSA)

National amendments (e.g. Germany): Regulation of

- Feed
- Food supplements
- Authorisation and certification of food-producing companies
- Prohibition of illness-related claims
- Consumer information

Regulation of the food sector: Food safety

Most parts from basic regulation (EC) No 178/2002

- Risk analysis (assessment, management, communication)
- Precautionary principle,
- Compliance with production standards,
- Feed and animal production included,
- EFSA responsible for
  - independent scientific advice on all aspects related to food safety,
  - preparing Community decisions,
  - operation of rapid alert systems, and
  - communication of risks.
- The Committee on Food Chain and Animal Health and Scientific panels subn „opinions“, carry out inspections...
- Member States may refuse authorisation for severe health threats

Regulation of the food sector: Consumer Information (1)

Consumer information

- Directive 2001/95/EC of 03.12.2001 (”Product safety”)
  - authorities may request all necessary information from all concerned parties
  - general obligation for information interchange, especially on risks

Labelling

- Directive 2000/13/EU
  - Explication of all ingredients
  - Introduction of list of most important allergenic ingredients, clear notice thereof on label

Regulation of the food sector: Consumer Information (2): Nutrition and health claims

Draft ”Regulation on nutrition and health claims made on foods” COM(2003) 424 final adopted by the European Commission on 16 July 2003

- Definitions: “nutrition claim”, “health claim”, “reduction of disease risk claim”
- Assumption: some characteristics (e.g. “low in cholesterol”) are already associated with health effects (e.g. good for heart health) “nutrition claim”
- Claims are only allowed on the basis of scientific evidence for a positive physiological effect of typical quantities.
- “Nutritional profiles” define characteristics that products need to allow specific claims, e.g. content in fat, sugar, salt.
- Health claims for “problematic” products (e.g. potato chips) will be forbidden.

Regulation of the food sector: Consumer Information (3): Nutrition and health claims

Draft ”Regulation on nutrition and health claims made on foods” COM(2003) 424 final adopted by the European Commission on 16 July 2003 (contd.)

- Nutrition claims are only allowed if they are noted in a list of (at the moment) claims.
- Health claims need explicit authorisation.
- Earlier authorised claims can be used without new authorisation.
- Risk reduction claims will not be forbidden anymore.

Regulation of the food sector: Criticisms on the draft nutrition & health claims regulation

(I) Contains provisions not consistent with its chosen legal base (Article 95 of the EC Treaty), → no need, no approximation of MS`s law

(II) Breach of Article 5 of the EC Treaty (the principle of proportionality and the principle of subsidiarity),

(III) Breach of Article 2.2 & 2.4 of the WTO Technical Barriers to Trade Agreement, → automatic prohibition of certain claims

(IV) Concerns vis-à-vis legal certainty → incorporating by reference provisions of Directives into a Regulation

(V) Breach of fundamental rights (Article 6 of the Treaty of the European Union and Article 153 (1) EC) → "a priori" prohibition of certain claims, without assessment of scientific evidence, right to a fair public hearing; right to freedom of expression; consumers' right to information

Source:
EFLA 2003 Position paper

International regulation of health claims

Japan: FOSHU licensing system (since 1991)

- Dietary supplements are included
- Approved FOSHU foods bear a seal of approval
- To achieve FOSHU status, companies submit a scientific dossier which includes
  - scientific documentation demonstrating the medical and nutritional basis for the health claim, incl. the recommended dose of the functional ingredient
- Approval is for the food or beverage as it is normally consumed.

USA (since 1991/1994)

- Law permits to use selected health-related claims, if claim can be scientifically supported

International level: Codex Alimentarius Commission

- "General Guidelines on the Use on Nutrition claims" (1997)
- Proposal on "Draft guidelines for use on nutrition and health claims"

Regulation of the food sector: Novel food/GM food

Regulation (EC) 1829/2003 on genetically modified foods and feeds, in force 18.05.2004

Regulation (EC) 1830/2003 on the traceability andl labelling of GMOs and products thereof, in force 2004

Directive 2001/18/EU on the deliberate release of GMOs and

Regulation (EC) 258/97 on "novel food" (= incl. GMOs or not used before May 15 th, 1997)

- Obligatory authorisation of products only by the Commission, valid for 10 yea
- Public information except of "confidential" information,
- Labelling based on production process, not on analysis of final product,
- No labelling for products of "indirect genetic modification" (e.g. animals fed with GMOs),
- No labelling of unintended or not avoidable content under 0.9%,
- Traceability from fork to farm over all steps.

Source:
Behr's Jahrbuch für die Lebensmittel-wirtschaft 2004. Hamburg: Behr

Regulation of the food sector: Food supplements

Directive 2002/46/EC of 10 June 2002
on the approximation of the laws of the Member States relating to food supplements

- 'Food supplements':
  - foodstuffs
  - concentrated sources of nutrients or other substances with a nutritional or physiological effect,
  - marketed in dose form (capsules, sachets of powder, ampoules of liquids...)
  - designed to be taken in measured small unit quantities;
- Only vitamins and minerals listed in Annex I of the Directive may be used for the manufacture of food supplements (no Phytoestrogens)
- Requires minimum amounts and maximum safe levels for normal use
- Labelling, presentation, warning

Conclusions for products with phytoestrogens (1)

- Most regulatory interventions exert both positive and negative impacts on the development and market introduction of new products
- The use of nutrition and health claims is seen as an opportunity to encourage the consumers to buy healthy foods.
- Foods and food supplements are highly regulated.
- To receive a marketing authorisation, the quality and safety of the product has to be demonstrated.
- To bring food products/supplements onto the market, not only the necessary minimal levels, but also the maximum intake levels have to be pointed out.

Conclusions for products with phytoestrogens (2)

- To use health claims, the positive effects of the product have to be establishe (incl. the need for the micro-nutrient in the target population).
- Phytoestrogens cannot be marketed as food supplements in the EU.
- Novel food status might be necessary (GMO, novel processes, novel molecu structure, no history of safe use, e.g. phytosterol-containing margarine "Becel pro-active").

AGROTECHNOLOGY & FOOD INNOVATIONS
WAGENINGEN UR

MAAS and the isolation of minor components like phytoestrogens

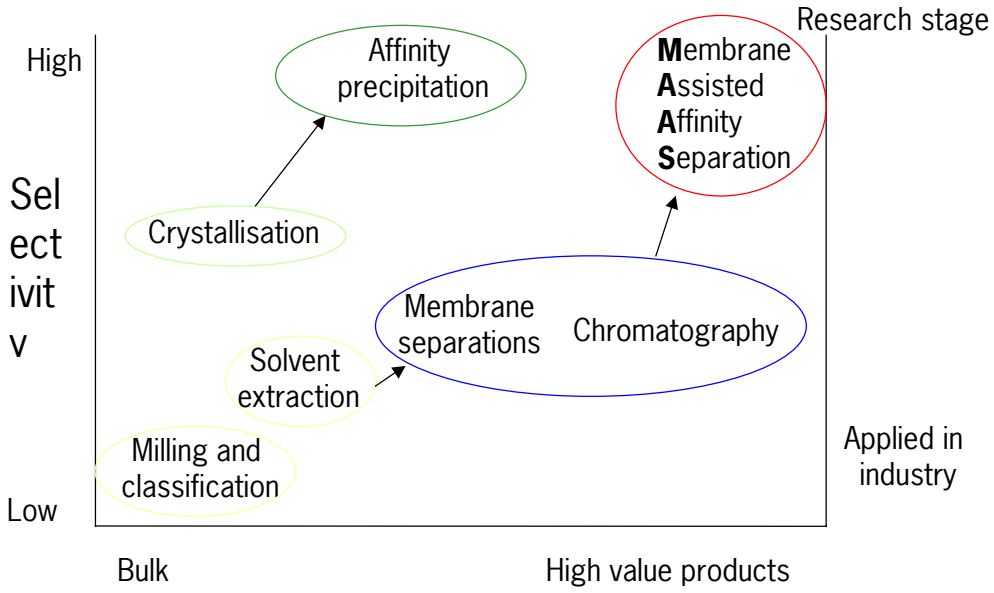
Karin Merck (A&F), Mike Litjens (A&F), Jan Henk Hanemaaijer (TNO-MEP), Joyce Schroot (A&F)

A&F: Agrotechnology and Food Innovations

Outline presentation

- Trends in separation processes
- MAAS
  - Principle
  - Isolation of lactoperoxidase from whey
    - Design parameters
    - Cost estimation
    - MAAS versus packed bed chromatography
- MAAS and phytoestrogens

Trends in separation processes



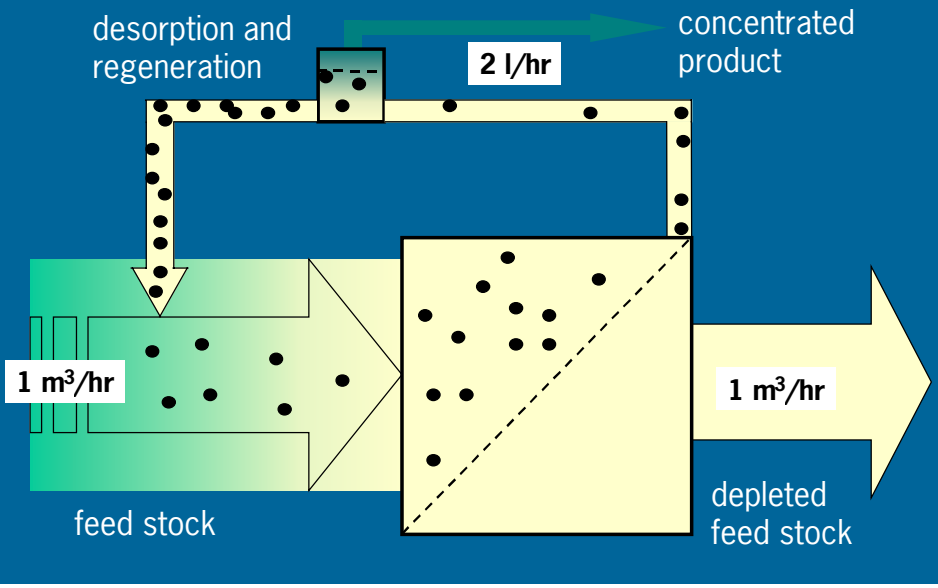
Trends in separation processes

- Needs in food industry
  - large volumes
  - applicable for low added value ingredients
  - selectivity

MAAS: what does it mean?

Membrane
Assisted
Adsorption
Separation

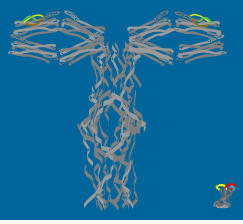
MAAS: how does it work?

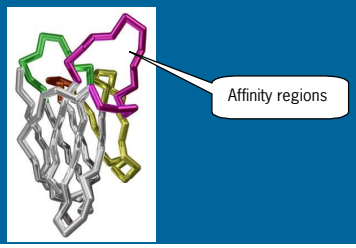


MAAS: how to get selectivity?

Selectivity: types of absorbent

- chromatography media like anion- or cation exchangers
- molecular imprinted polymer
- industrial affinity ligands





MAAS: current status

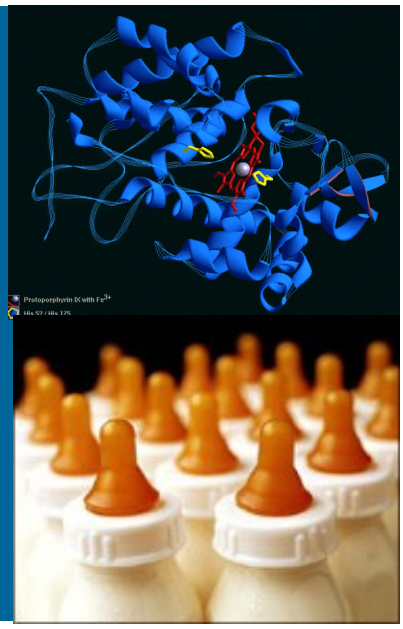
MAAS-concept is tested for several applications

Removal of minor components (amino acids, benzene, caffeine)
Removal of heavy metals
Removal of dyes from textile wastewater
Isolation of lactoperoxidase from whey

MAAS for water purification :TNO-MEP patent WO9637272

MAAS: case study (1)

- Target molecule:
  - lactoperoxidase
  - applications based on (among others) antimicrobial and antiviral activity
    - » baby food
    - » food supplements
    - » cosmetics
    - » oral care
  - cation exchange chromatography



MAAS: case study (2)

Source

whey powder or skimmed milk

Whey powder composition

Moisture	3-4 %
Lactose	70-75 %
Fat	0 %
Total protein	10 -13 %
Ash	7 - 12 %
Lactoperoxidase	0.5 % of the total protein

MAAS: case study (3)

Isoelectric points of whey proteins

lactoperoxidase (LPO)	9.6
lactoferrin (LF)	8.0
βLG	5.2
αLA	4.7 - 5.1
BSA	4.9
Ig	5.8 - 7.3

At pH 6.7 Adsorption on cation exchange beads
Desorption using 0.15 M NaCl

DMV Campina patent WO13676 (1993)

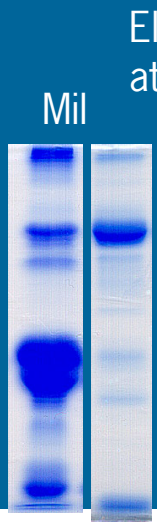
MAAS: case study (4)

Design parameters

LPO from whey using Source 30 beads

Kinetics of adsorption anddesorption
< 2 minutes and < 0.5 minutes

Purification factor
213

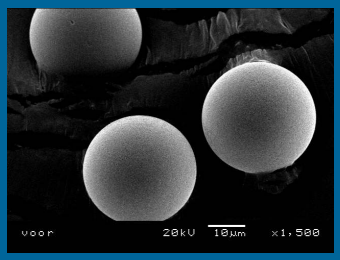


MAAS: case study (5)

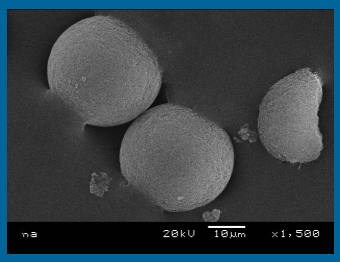
Bead stability

Mechanical stability of the porous beads

SEM pictures of Source 30 after 65.000 passages



before



after

Thermal and chemical stability of affinity ligand

MAAS: case study (6)

Assumptions cost estimation

– 250 m^3/hr 0.2 gr LPO + LF per liter skimmed milk
– 400.000 kg dry LPO/LF per year
– value of feed and depleted feed €0,20 per liter

MAAS: case study (7)

Cost estimation	€/kg product
Investments	1,90
Variable costs	
electricity, membranes, salts	0,90
adsorbent (€2500/l)	4,80
Total costs	7,60

MAAS: case study (8)

- Return of Investment

  €200/kg ➜ 14 days
  €20/kg ➜ 9 months

  Current market value of LPO and LF (95+%): 250 - 750 € per kg

MAAS: case study (9)

MAAS versus packed bed chromatography

Process characteristic	MAAS	PBC
Protein production	49 kg/hr	1 - 7 kg/cycle
Floor space [m^2]	20	150 - 200
Required # units	1	80
Adsorbent [kg]	125	1200- 2500

MAAS and phytoestrogens

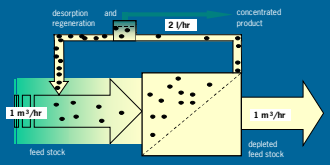
Identify sources and ingredients for which MAAS is potentially interesting

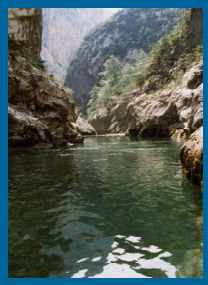
- from raw materials or co-products of food processing
- phytoestrogens such as isoflavones and lignans
- bioactive peptides
- natural colourants




MAAS and phytoestrogens

- MAAS using an affinity ligand for the removal of the estrogenic compound 17α-ethinylestradiol from waste water





Last question

"Phytoestrogen" is more than soy-derived isoflavones: Additional sources, structures, and isolation methods

Melanie Stürtz, Gerold Jerz, Nils Köhler, Oliver Nehring, and **Peter Winterhalter***
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Sources of Phytoestrogens

Plants contain several different families of natural products among which are compounds exhibiting weak estrogenic or antiestrogenic activity [1-3]. Best studied representatives among these so-called phytoestrogens are the soy-derived isoflavones as well as lignans from flaxseed. However there are additional classes of phytoestrogens which also deserve our attention. From hops (Humulus lupulus L.), a series of prenylated flavonoid phytoestrogens has been isolated together with the potent anticarcinogenic chalcone xanthohumol. Occurrence of such prenylated flavonoids is by far not restricted to H. lupulus, in several other plants prenylated flavonoids are present. Another class of phytoestrogens are the stilbenes and red wine or extracts thereof are prominent sources for e.g. 3,5,4´-trihydroxystilbene (resveratrol). From licorice root licochalcone-A can been obtained which also showed estrogenic and antitumor effects in cell cultures. Finally coumestanes a class of phytoestrogens which exhibit a close structural similarity to isoflavones should be mentioned. Typical sources for coumestanes are alfalfa, soybean and clover sprouts.
After an overview on the above mentioned classes of phytoestrogens, methods for the preparative isolation of pure standard compounds are presented.

Preparative Separation using Countercurrent Chromatography

Preparative separations of in the 10-100 g scale using High Speed Countercurrent Chromatography (HSCCC) and Low Speed Rotary Countercurrent Chromatography (LSRCCC) are today feasible. During a single separation step gram amounts of pure compounds, which can then be applied to further biological studies, are obtained. Countercurrent chromatography is an automated version of liquid-liquid extraction, comparable to the repeated partitioning of an analyte between two immiscible phases by vigorous mixing in a separatory funnel. The real breakthrough of CCC came with the invention of the Coil Planet Centrifuge that was introduced by Y. Ito in 1981 [4]. Today this technique is known as High-speed Countercurrent Chromatography (HSCCC). HSCCC separation takes place in a so-called "multi-layer coil" that is made by wrapping an inert Teflon tubing around a holder in multiple layers. Consequently the technique is also known as Multilayer Coil Countercurrent Chromatography (MLCCC). The tubing usually has an inner diameter between 1.6 and 2.6 mm and the length can reach 160 m. Multiple coils can be connected in series to increase the total volume of the instrument and the sample capacity. During separation the coil is rotated in a planetary fashion; it rotates at 800 to 1000 rpm around its own 'planetary' axis and simultaneously around a parallel 'solar' axis. This planetary rotation has two effects:
(i) The rotation creates a fluctuating acceleration field which enables vigorous mixing of the two phases followed by settling within the coil. In areas of the coil which are close to the centre of rotation the force field is weak. As a consequence, the phases are mixed. At a further point of their orbit, when they are far away from the centre of

rotation, the force field becomes stronger and the two phases are separated. Alternate mixing and settling is repeated with each rotation and in this way up to 50 000 partitioning steps per hour can be achieved.
(ii) Rotation of the coil also enables retention of stationary phase. During rotation of a coil filled with two immiscible liquids it can be observed that the two phases move towards opposite ends of the coil, known as head and tail. Generally the less dense phase displaces the heavier phase towards the tail, but the orientation is also known to be influenced by viscosity and interfacial tension. This phenomenon, known as hydrodynamic equilibrium, requires to choose the elution mode carefully but it also gives the analyst the freedom to select either the lighter or the heavier layer as mobile or stationary phase. When the heavier phase is selected as the mobile phase, the proper elution mode is head to tail and the mobile phase is introduced from the head of the system. By choosing the lighter phase as the mobile phase the elution order of the compounds is reversed, the correct elution mode is tail to head and the mobile phase is pumped into the tail of the system.

A different approach is used in Low-Speed Rotary Countercurrent Chromatography (LSRCCC). In LSRCCC a cylindrical column rotates slowly around a single axis. Du and coworkers [5-7] showed that the use of special convoluted tubing (e.g. with 8.5 mm inner diameter) enabled sufficient retention of stationary phase at a rotational speed of only 5-100 rpm. The instrument is readily scaled up by using longer columns and/or by increasing the inner diameter of the convoluted tubing. Instruments that are suitable for separation in the kg-scale are under development [8].

Examples for Phytoestrogen Isolation using Countercurrent Chromatography

Details on the isolation of lignans from flaxseed and isoflavones from soybean can be found in the literature cited [9,10].

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Isolation of new lignans from sesame agricultural wastes using resin adsorption technology.

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Sesamum indicum (Peadaliaceae) is a well know source of lignans, which are molecules generally considered as potential phytoestrogens.
The process used by the food industry for the purification of sesame seeds produces big quantities of the sesame coat (pericarp) which is an agricultural waste, used only for animal breeding. Although the chemical consistency of the sesame pericarp has never been studied in detail, it is known that it is also an interesting source of lignans. Additionally the sesame coat contains 5 times less oil than the sesame seeds which are very rich in oil (around 50 %), and consequently the lignans can be more easily isolated.
The present study concerns the development of a methodology for the efficient obtainment of extracts rich in polyphenols from the sesame pericarps and the chemical investigation the contained lignans.
The first step of the followed procedure was the extraction of the dried pericarp using solvents of increasing polarity: cyclohexane and ddichloromethane in order to remove the oil efficiently and then methanol for the extraction of polyphenols. The methanol extract contained a high proportion of polyphenols but also important quantitities of sugars and residual lipids. In order to enrich the polyphenol concentration, the dried methanol extract was diluted with warm water and this aqueous solution passed through a column containing XAD-4 resin, which selectively adsorbed the polyphenolic compounds. Then, the polyphenols were removed from the resin with methanol, which after evaporation, furnished an enriched polyphenol extract.
This dry extract was submitted to several chromatographic separations that finally led to the isolation of 5 known lignans: sesamine, pinoresinol, lariciresinol, olivil and 3-methoxylariciresinol and three new lignans.
All comounds were identified by means of spectral data (^{1}H NMR ^{13} C NMR, 2D NMR: COSY, HMBC, HMQC and NOESY).
The biological activity of the extract and of the isolated lignans is currently under investigation.

Isolation of natural antioxidants from *olea europea* leaves, fruit and wastewaters

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The Mediterranean diet, rich in fresh fruit and vegetables, has been associated with a low incidence of cardiovascular disease and cancer, partly because of its high proportion of bioactive compounds such as vitamins, flavonoids and polyphenols. The major lipid component of such a diet is the druped-derived olive oil. Also several studies have shown that olive leaf extract has beneficial effect on human health. The bitter compound oleuropein, the major constituent of the secoiridoid family in the olive (*Olea europea L.*) trees, has been shown to be a potent antioxidant endowed with anti-inflammatory properties.
The purpose of this study was to isolate the main bioactive compounds present in olive leaves, olive fruit and olive mill waste waters and examine their antioxidant activity and their effect on osteoporosis.
The concentration of the polyphenolic compounds in olive oil ranges from 50 to 1000 μg/g ro foil depending on the olive variety and the extraction system. This amount of antixoxidants in the olive oil is 1-2% of the available pool of antixodants in the olive fruit. The rest is lost with the wastewater (approximately 53 %) and the pomace (approximately 45 %). The high polyphenol content of the wastewater is the major factor of the environmental problems caused by the olive mills.
We designed and developed at pilot scale a system for the treatment of the olive oil mills wastewater with the aim to recover the polyphenols and reduce the environmental problems. The olive oil mill wastewater (OMWW) treatment procedure includes the pass of filtered wastewater through a series of specialised adsorbent resins (XAD-4, XAD-7) in order to achieve the deodouring and decolourisation of the wastewater and the recovery of the polyphenols and lactons content.

Pure oleuropein obtained from olive leaves and the extract of OMWW, which is rich in polyphenols and lactones, both have shown interesting anti-oxidant activity.

Review of the extraction procedures for analytical methods used in the determination of phytoestrogens in food and environmental samples.

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In view of the possible health benefits of phytoestrogens, an the evidences suggesting that phytoestrogens might protect against various cancers, particularly against breast and prostate cancer, makes that their use as complements in diet and in health care practices has grown highly.
But on the other hand, the use of some phytoestrogen at levels far higher than those seen in food for a long period of time, has been related with the contrary effects, as the possibly increase cancer risks. Moreover, their potential as environmental endocrine disrupters it should be taken into account. Another aspect to be considered is that the main difference between phytoestrogens as beneficial health diet components and dangerous hormones, quite capable of promoting cancer, is the dose. So, there is a need to develop analytical methods at low level of determination. In this respect, novel, rapid and more sensitive analytical approaches are needed in order to evaluate their effects.
One of the most relevant aspects for the analysis of phytoestrogens and their metabolites is the extraction and isolation, because it is particularly difficult given the similarity of structures and chemical properties, as well as the range of matrices in which they are found. Phytoestrogens and their metabolites are generally present in parts per billion to parts per million concentrations in plants, solid and liquid foodstuffs as well as in biological matrices such as plasma, serum, urine and faeces.
Phytoestrogens must be isolated from the major constituents of these matrices before quantification. The type of matrix, phytoestrogen and the analytical method determine which isolation or extraction procedures are required.
In this presentation isolation and clean up protocols for phytoestrogens in food and environmental matrices will be review.