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Recovery of isoflavones from red clover flowers by a membrane-based process ☆

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Abstract

Isoflavones in red clover flowers were extracted and recovered using a new process that mainly comprised of ethanol extraction, membrane processing, micelle formation and drying. To obtain maximum isoflavone extraction an ethanol concentration between 40% and 50% in water was found to be optimal. The extracted isoflavones were processed by ultrafiltration for preliminary purification, and then concentrated by reverse osmosis. As ethanol was removed by evaporation, micelles were formed in the reverse osmosis retentate, which was dried to yield an isoflavone-enriched product. This product contained about 9% isoflavone highlighting its potential use as a direct nutraceutical supplement.

Keywords: Isoflavones; Red clover flower; Extraction; Membrane and process

Industrial relevance: Isoflavones present in agricultural biomass are utilized as source of functional food supplements. Extraction and refining of isoflavones involves several steps and the process is very energy intensive. This work reports an interesting approach for extraction and refining of isoflavones by developing a new process, which is energy efficient and gives a final product, which contains sufficiently high amounts of isoflavones for consumer applications.

1. Introduction

Isoflavones from plant sources are typically nutraceutical products for their distinctive health benefits, which include the reduction of risk of osteoporosis and cardiovascular diseases (Adlercreutz & Mazur, 1997; Anderson & Carner, 1997), prevention of certain hormone-related cancers (Adlercreutz, 1995; Barnes, 1997; Wei, Wei, Frenkel, Bowen, & Barnes, 1993), and relief of menopausal symptoms (Kuzer, 2000; Liu et al., 2001). Although soybeans are currently the main source of isoflavones, lately red clover has received increasing attention for the high isoflavone content in its various parts. The isoflavone composition of red clover, however, is quite different from that of soybeans. The predominant components in red clover are biochanin A and formononetin (Chen, Jin, Tao, & Qin, 2004; Liu et al., 2001; Xu, Lamb, Layton, & Kumar, 2005), which are methyl ether derivatives of genistein and daidzein, respectively, whereas in soybeans genistin and daidzin, the respective glucosides of genistein and daidzein, are the principal isoflavones. However the glucosides of these, sissotrin and ononin, were not detected. The findings were similar to those reported by some previous researchers (Chen et al., 2004; Liu et al., 2001; Vetter, 1995), while contradicting others that claimed the presence of glucosides and esterified glucosides of biochanin A and formononetin in various parts of red clover (Toebes, de Boer, Verkleij, Lingeman, & Ernst, 2005). The variations in isoflavone composition may be due to varietal diversity as well as differences in growth and storage conditions of the plant. Despite the compositional difference, the bioavailability of isoflavones from these two plant sources is similar (Tsunoda, Pomeroy, & Nestel, 2002). Nutritional studies also indicate that red clover-derived isoflavones do not show any estrogenic activity, thus making them superior to the soy-based isoflavones for use in hormone replacement therapy (HRT) (Atkinson et al., 2004).

Therefore there is a potential demand for isoflavones recovered from red clover as concentrates or isolates. These products can be prepared using methods developed for isoflavone extraction and isolation from soybeans, which often use large quantities of organic solvents in combination

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with various chromatographic techniques (Chang, 2002). It is costly and time consuming to make the high quality products using these processes. More economical and efficient processes have yet to be developed which are suitable for the recovery of isoflavones from red clover. Xu et al. (2005) described a simple scheme to recover isoflavones from red clover flowers as a concentrate, consisting of four main steps: extraction, adsorption, elution and drying. This process was able to produce a final product with an isoflavone content of approximately 20%, which accounted for more than half of the total amount of isoflavones in red clover flowers. The product could be directly used to make pills as isoflavone supplement, or further purified to obtain isoflavone isolates. This process, however, left behind a high volume of waste stream as a result of the use of large quantities of water, which may pose an environmental concern if not treated properly.

Alcohols, particularly methanol, have been extensively used by previous researchers to extract isoflavones from red clover for analytical purposes. In the present study, a new approach was investigated for isoflavone processing, where ethanol was chosen as the solvent for extraction due to health concern. The extract was ultrafiltered before concentration by reverse osmosis (RO). After the removal of ethanol from the concentrated solution, micelles were allowed to form by refrigerating and this concentrate was subsequently dried. The resulting product was an isoflavone enriched powder.

2. Materials and methods

2.1. Extraction of isoflavones from red clover flowers with ethanol

Dried red clover flowers were purchased from Frontier Natural Products (Norway, IA). They were ground in a Warring blender (Warring Commercial, Torrington, CT) for 2 min, and well mixed in a pail. Subsequent experiments for isoflavone extractions were performed with batches of 90 g ground material.

Each batch was mixed with an aqueous ethanol solution at a solvent-to-flowers ratio of 40 : 1 in a 4 l beaker, and the ethanol concentration for extraction was varied from 0% to 100%. The slurry was stirred on a hot plate stirrer at room temperature. The beaker was covered with cling wrap to minimize ethanol loss by evaporation. Replicate samples of 3 ml were taken from the extraction solution at intervals of 15 min for the first hour and 30 min thereafter, and then prepared for isoflavone analyses.

2.2. Isoflavone recovery by ultrafiltration (UF) and reverse osmosis (RO)

Ground red clover flowers were first extracted with 40% ethanol solution in the same manner as above. After straining through Tyler No. 18 and No. 400 sieves, the extracts from four different batches were combined and ultrafiltered in a PLAC Prep/Scale TFF Cartridge (Millipore Ltd., Bedford, MA) of regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 1000 Da. A trans-membrane pressure of 550 kPa was applied to give a permeate flux of 7.0 l/m^2 h. Ultrafiltration was continued until the feed volume was reduced by about 90%. A thin-film commercial RO membrane (Desal-3 GE Osmonics, Vista, CA) with a salt rejection of 98% was used for concentrating the permeate from UF. A laboratory scale plate and frame system with a membrane area of 1050 cm^3 was used at a trans-membrane pressure of 1800 kPa at ambient temperature with a feed recirculation rate of 192 l/h and a permeate flux of 15 l/m^2 h. The UF permeate was concentrated 10 to 15 times by this RO process. The concentrate from RO was evaporated under vacuum at 60 °C in a Laborota 4000 evaporator (Heidolph Instruments, Cinnaminson, NJ) to remove ethanol, and the remaining portion was lyophilized for 72 h using a FreeZone 1 l freeze dryer (Labconco Corp., Kansas City, MO). The residue of red clover flowers after ethanol extraction was dried at 105 °C overnight in an oven.

2.3. Isoflavone recovery by RO and micelle formation

In this experiment, the alcoholic extract of red clover flowers was membrane processed using UF and RO units as above. Most of the ethanol in the RO retentate was removed by evaporator while the residue was placed in a ventilated oven at 90 °C until its volume was reduced to about 1/3 that of the original RO retentate. This concentrated residue was then left in a refrigerator at 4 °C for 24 h to allow micelles to form and settle. The supernatant was decanted the next day, and the micelles at bottom of a beaker were collected in water. These were freeze dried for 48 h to make a powdery product.

2.4. Isoflavone analyses

Each aqueous sample was prepared by mixing it with an equal amount of HPLC-grade methanol containing 2% glacial acetic acid (v/v). All ethanol samples were analyzed as is, and solid samples were analyzed as reported earlier (Xu et al., 2005). For the determination of isoflavone concentration, a HPLC-based method was used according to the parameters set by Klump et al. (2001). The key instrument was a Hewlett Packard 1100 HPLC system with a Zorbax SB-C18 reversed-phase column (250 × 4.6 mm i.d.) and a diode array UV detector (Agilent Technologies, Wilmington, DE). The isoflavone standards were purchased from Indofine Chemical Co. (Somerville, NJ). All analytical results of isoflavone determination were expressed as μg/g for consistency.

3. Results and discussion

3.1. Extraction of isoflavones from red clover flowers

The results of isoflavone analyses for this batch of red clover flowers were reported in the work of Xu et al. (2005), including a total isoflavone content of 2590 μg/g, of which biochanin A and formononetin were the major components. The findings were similar to those reported by some previous researchers (Chen et al., 2004; Liu et al., 2001; Vetter, 1995).

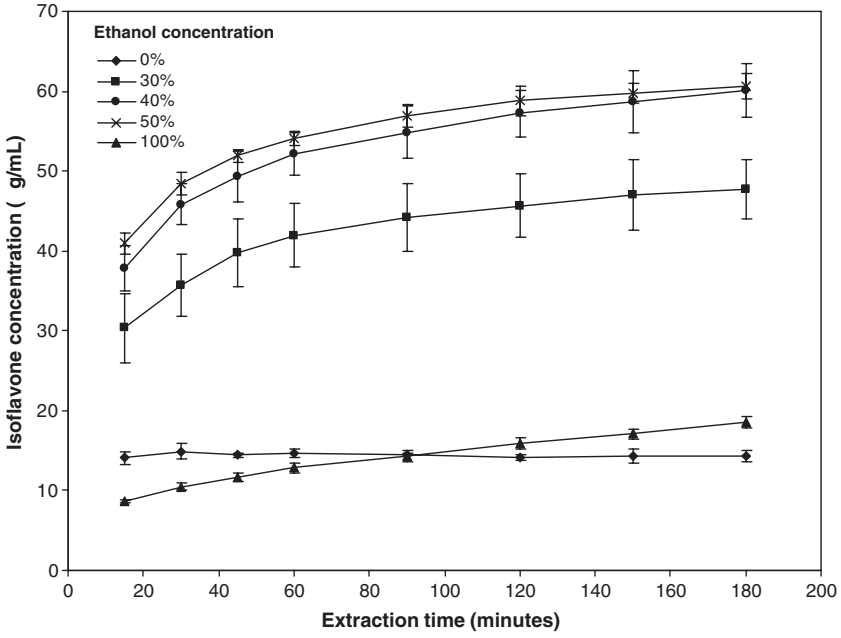

Fig. 1. Extraction of isoflavones from red clover flowers at different ethanol concentrations. Error bars are based on duplicate runs.

In order to investigate the effect of ethanol concentration on the extractability of isoflavones, ethanol was mixed with water at different ratios by volume. The extraction curves in Fig. 1 indicate that similar to alkaline extraction (Xu et al., 2005), ethanolic extraction of isoflavones also took about 2 h to reach equilibrium in the cases of 30%, 40% and 50% ethanol concentration (v/v) in water, where the isoflavone concentration in the extract increased steadily in the first 2 h and then began to level off. Fig. 2 shows a "bell" shaped extractability curve with a peak of nearly 90% extractability between 40% and 50% ethanol concentration. It is noted that extraction with either water alone or pure ethanol resulted in low extractability of isoflavones (~20%). Generally poor extraction results were expected with water as these isoflavones were nearly insoluble in water; it was surprising to observe a similarly low extractability with pure ethanol, considering that isoflavones were highly soluble in ethanol, and ethanol was used effectively in the work of Xu et al. (2005) to elute the isoflavones adsorbed on polyvinylpolypyrrolidone (insoluble PVP). This observation could not be explained on the basis of viscosity change of

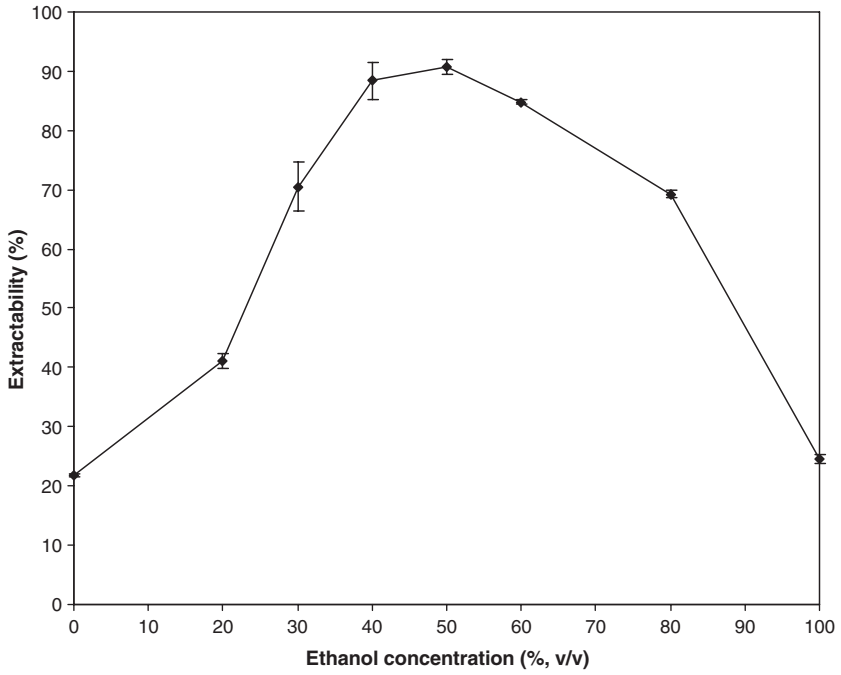

Fig. 2. Effects of ethanol concentration in water on the extractability of isoflavones. Error bars are based on duplicate runs.

Table 1
Isoflavone distribution among streams of UF and RO

Stream	Mass (g)	Isoflavone content (μg/g)	Isoflavone amount (mg)	%
Red clover	360	2.59×10^3	932	100
Dried spent residue	245	676	166	17.8
Retentate of 1k ultrafiltration	953	96.8	92.3	9.9
Permeate of RO	1.14×10^4	17.7	202	21.7
Dried retentate of RO	55.1	7.17×10^3	395	42.4
Unaccountable[a]	n.a.[b]	n.a.	76.7	8.2

[a] All product streams minus starting material (red clover flowers).
[b] Not applicable.

aqueous ethanol solution as a result of alcohol concentration change, since the viscosity reached a maximum at 50% ethanol (Marcus, 2002). Instead, the polarity change of the solution might have played a critical role in affecting the extraction, according to the theory of preferential solvation proposed by Dawber, Ward, and Williams (1988) in the study of reaction rates in mixed solvents using pyridinium betaine. The concept of an excess polarity was introduced to represent the departure of polarity from a linear relationship in a binary solvent mixture, and a positive excess polarity indicated preferential solvation of a solute by the more polar component of the solvent, whereas a negative one represented preferential solvation by the less polar component. In the present study the amphiphillic nature of isoflavones may have made these compounds preferentially solvated by ethanol in the regions of low ethanol concentrations (<20%), where the alcohol was not abundantly available. However at high ethanol concentrations (>80%), where water was scant, the isoflavones were preferentially solvated by water. It was likely due to this paradox that low isoflavone extractability was experienced on both ends of the ethanol concentration range, hence the "bell" shape of the extractability curve. This knowledge was critical to isoflavone extraction from red clover using aqueous ethanol solutions, and apparently the optimal ethanol concentration was between 40% and 50% so as to obtain maximum yield of isoflavones.

Table 2
Isoflavone distribution among streams of UF, RO and micelle formation

Stream	Mass (g)	Isoflavone content (μg/g)	Isoflavone amount (mg)	%
Red clover flowers	360	2.59×10^3	932	100
Dried spent residue	259	677	175	18.8
Retentate of 1k ultrafiltration	925	84.3	78.0	8.4
Permeate of RO	1.12×10^4	13.4	150	16.1
Supernatant after micelle formation	526	267	140	15.0
Dried micelles	3.70	8.88×10^4	329	35.3
Unaccountable[a]	n.a.[b]	n.a.	−60.0	−6.4

[a] All product streams minus starting material (red clover flowers).
[b] Not applicable.

3.2. UF of ethanol extract with 1k membrane

The ethanol extract of red clover contained various other components besides isoflavones. An UF step was introduced to clarify the feed by eliminating proteins and polypeptides, and to reduce the amount of other impurities for further membrane processing using RO. As the molecular weights of the isoflavones in this study were below 1000, a membrane cartridge was selected to retain compounds with molecular weights greater than 1000 in the extract, while permeating most of the extracted isoflavones. A typical UF process could be described by the following equation (Cheryan, 1986):

$$\frac{C_f}{C_0} = \left(\frac{V_0}{V_f}\right)^R = (\mathrm{CF})^R$$

here C_0 is the isoflavone concentration in the ethanol extract of red clover, C_f the isoflavone concentration in the final retentate of UF, V_0 the volume of the ethanol extract, V_f the volume of final retentate, R the rejection coefficient of solute, and CF is the concentration factor. The value of R varying between 0 and 1 indicates the permeability of solute by the membrane, with 0

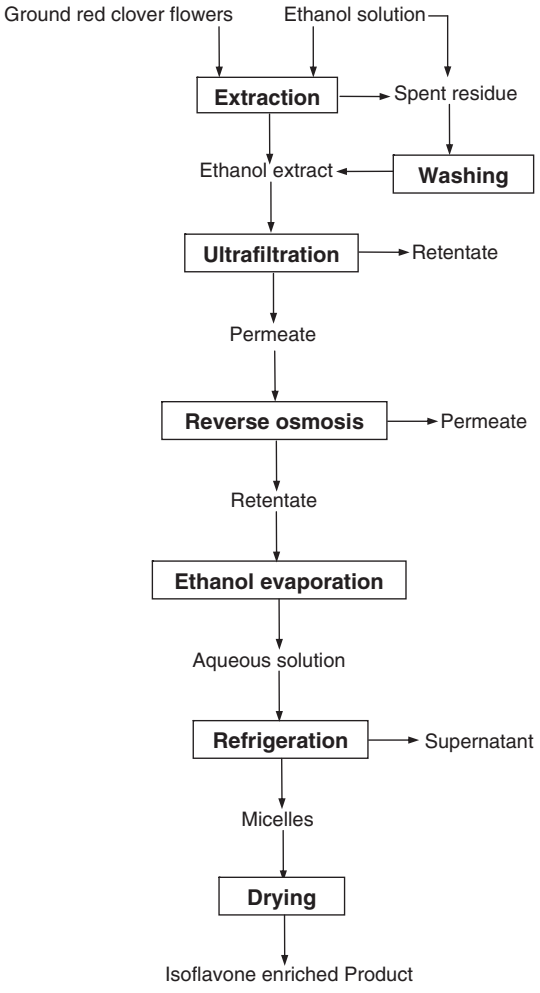

Fig. 3. Flow diagram of a process to make an isoflavone enriched product from red clover flowers.

for free passage and 1 for total rejection. In an UF experiment, 14.3 l of the ethanol extract with an isoflavone concentration of 56.1 μg/ml was concentrated to 1.0 l of retentate containing 91.4 μg/ml isoflavones. Using the above equation, the overall R value of the isoflavones in this case was estimated to be 0.18 for the membrane. In the work of Xu, Lamb, Layton, and Kumar (2004) on soy isoflavones, the R values for the same membrane cartridge were determined to be 0.22 and 0.05 for glucosides and aglycones, respectively. The two major isoflavone components in red clover, biochanin A and formononetin were larger in molecular weight than those isoflavone aglycones in soybeans such as genistein and daidzein, but smaller than the isoflavone glucosides like genistin and daidzin. Therefore, it was reasonable that the red clover-derived isoflavones exhibited an overall R value between those of soy-based isoflavone aglycones and glucosides since the separation by UF was essentially based on difference in molecular size. By the estimated R value and the above characteristic equation of UF, it was easily calculated that with a concentration factor of 10, more than 85% of the isoflavones in the ethanol extract could be permeated, thus rendering any further membrane processing such as diafiltration (DF) unnecessary for achieving higher yields. However, as a result of a higher viscosity of the ethanol solution (~2.3 mPa·s) than that of water (~1.0 mPa·s), the permeate flux was reduced to about 7.0 l/m^2 h, as compared to 10 l/m^2 h in the case of water (Xu et al., 2004).

3.3. Isoflavone recovery by RO and micelle formation

The above permeate of UF contained approximately 70% of the total amount of isoflavones in red clover flowers, and it was concentrated by RO to recover these isoflavones. As observed in UF, the permeate flux of RO was also decreased to about 15 l/m^2 h in comparison to 24 l/m^2 h attained with water (Xu et al., 2004), again due to the higher viscosity of the ethanol solution. Table 1 shows the isoflavone distribution for the whole process with red clover flowers as the starting material. It was found that even after ethanol washing there was still about 18% of the total amount of isoflavones remaining in the dried spent residue, which was likely the result of the residue holding an amount of extract up to five times its dry weight. A high concentration factor of 15 was used for UF in this run, thus resulting in less than 10% of the total amount of isoflavones in the retentate. However, a concentration factor of 10 for the RO process step led to a loss of 15–20% isoflavones to its permeate, which was much greater than the approximately 5% value in the case of aqueous processing of soy isoflavones (Xu et al., 2004). The presence of ethanol might have affected the interactions between isoflavones and the RO membrane and facilitated the permeation of isoflavones through the membrane. The RO retentate had a recovery of isoflavone in excess of 40% of the total amount, and after the removal of ethanol and followed by freeze drying, it gave a brown powdery product that contained 7.2 $\times 10^3$ μg/g isoflavones, or 0.72%. Although the mass yield of this product was relatively high, its quality was unfortunately low in terms of isoflavone content. In order for it to be qualified for any applications, further purification was necessary.

In another run a different approach was taken following the RO step. After ethanol from the retentate was removed in the evaporator and the oven, the remaining aqueous portion was allowed to stand for a period of 24 h at 4 °C. This concentrate became hazy and the precipitate slowly formed a layer, which subsequently settled at the bottom of the container. This material appeared to be micelles containing large amount of isoflavones, and when freeze dried it yielded a light brown powder with an isoflavone content of 8.9×10^4 μg/g, or 8.9% (Table 2). The formation of the micelles was probably due to the hydrophobic nature of red clover-derived isoflavones over most of their structures. Therefore, as the extracted isoflavones were highly concentrated by RO to exceed the "critical micelle concentration" (CMC), they became unstable in water as the ethanol was removed. The isoflavone molecules then aggregated by hydrophobic interactions to form micelles, which produced a sticky precipitate containing the isoflavones and other hydrophobic components from red clover. The supernatant after the micelle formation had an isoflavone content of 267 μg/g, which could be used as an approximate value of CMC. Based on these findings, a process has been developed (Fig. 3) to make a product with an isoflavone content of nearly 9.0%, which may be used as an isoflavone supplement without further processing. The isoflavone recovery of this product was, however, only about 1/3 of the total amount in red clover flowers (Table 2). This low recovery seemed inevitable when the isoflavones were distributed amongst a number of product streams. However, product recovery could be substantially improved if the RO permeate was reused for isoflavone extraction of the next batch, and some of the product streams such as the UF permeate were combined with the extract from the next batch. Also, this approach would be both economically and environmentally beneficial to the process.

4. Conclusions

It was concluded that the optimal ethanol (v/v) concentration in water for extraction of isoflavones from red clover flowers was between 40% and 50%, where extractabilities of higher than 90% were obtained. Pure ethanol or water alone, however, resulted in much lower extraction of isoflavones. The extracted isoflavones were highly permeable through a 1000 Da molecular weight cut-off membrane, and could be concentrated by RO. Micelles were formed in the concentrated isoflavone extract, as ethanol was removed by evaporation. A product containing almost 9.0% isoflavones, which represented about 35% of the total isoflavone amount in red clover flowers could be made by drying the micelle containing liquid.

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