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Pretreatment of milk thistle seed to increase the silymarin yield: An alternative to petroleum ether defatting

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Abstract

Milk thistle (*Silybum marianum* L.) seed meal is extracted for the flavonolignans, silychristin, silydianin, silybinin A, silybinin B, isosilybinin A and isosilybinin B, which are collectively known as the silymarin complex. To obtain the flavonolignans, the meal is usually treated with successive washes of petroleum ether to remove the lipids, followed by extraction of the flavonolignans with ethanol. This work examines the possible replacement of petroleum ether and ethanol by water or other aqueous solutions in these processes. To replace petroleum ether, pretreatments with 1.2% NaOH (w/w), 1.5% H_2SO_4 (w/w), 2% $NaHCO_3$ (w/w), 0.14% cellulase and water were investigated. Of these pretreatments, 1.5% H_2SO_4 and water produced similar flavonolignan yields as petroleum ether. Results established that pretreating the milk thistle seed meal with 1.5% H_2SO_4 (w/w) at 50 °C for 18 h could replace the petroleum ether pretreatment. In addition, it was shown that similar amounts of flavonolignan could be recovered with a 1.5% H_2SO_4/water (100 °C) extraction as with a petroleum ether/ethanol extraction. Although cellulase pretreatment was not examined extensively, significant advances in cellulase effectiveness and cost have occurred in the past few years by companies such as Genencor International and Novozymes. These advances should help to make enzyme use for cellulose conversion, as well as extraction pretreatment, technically and economically feasible.

Keywords: Milk thistle; Pretreatment; Silymarin; Flavonolignans; Water extraction

1. Introduction

Phytochemicals are extracted from plant materials to service the pharmaceutical and the dietary supplement industries. Processes such as supercritical fluid extraction (SFE) (Femenia et al., 2001), pressurized fluid extraction (PFE) with enhanced solvent diffusivities (Benthin et al., 1999) or leaching with generally regarded as safe (GRAS) solvents provide efficient extraction processes for these compounds (Tanko et al., 2005). To increase the extraction yield, the biomass can undergo a physical, chemical or biological pretreatment (Lynd et al., 1999), which breaks down the rigid cell wall matrices, thereby resulting in a more efficient extraction. Physical pretreatment, such as grinding or freeze explosion, can be used as the sole pretreatment step or as a treatment prior to additional chemical or biological pretreatment. Chemical pretreatments with dilute sulfuric acid (Allen et al., 2001), ammonia (Belkacemi et al., 1998), dilute sodium hydroxide (Li et al., 2001) and water (Allen et al., 2001) have been used in the conversion of cellulose to sugars, particularly when enzymatic hydrolysis is used. In addition to chemical or physical pretreatment, hydrolytic enzymes have been used as pretreating agents, acting on cell walls and breaking down structural integrity, thereby increasing the surface area of the material. As an example, cellulose pretreatment was found to be effective in releasing lutein from marigold flowers (Barzana et al., 2002).

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Extracts of milk thistle (*Silybum marianum* L.) seed have a long tradition for treating liver ailments (Flora et al., 1998). In 2005, milk thistle seed products placed in the top ten of dietary supplements, at about $8.3 million (Herbalgram, 2006). The seeds contain the silymarin complex, which is composed of the six flavonolignans silychristin (SC), silydianin (SD), silybinin A (SA), silybinin B (SB), isosilybinin A (ISA) and isosilybinin B (ISB) (Wallace et al., 2003a). Because milk thistle seed contains 20–25% (w/w) lipid (Hamid et al., 1983; Carrier et al., 2002), the seeds are usually extracted with petroleum ether to defat the seed prior to flavonolignan extraction (Benthin et al., 1999). Petroleum ether is regulated as a volatile organic compound (VOC), is expensive and, after its use, requires recovery or disposal. Additionally, the trace quantities of petroleum ether still present in the final extract need to be removed to meet consumer acceptability. Thus, alternative pretreatment techniques are sought. Kahol et al. (2001) ground and froze milk thistle seed prior to flavonoligan extraction, but cryogenic treatment is an expensive process. Because flavonolignan extraction with water has been successful on both defatted seed and seed that had not been defatted (Wallace et al., 2003b; Duan et al., 2003), an attempt was made to interface novel pretreatment techniques with water extraction. Thus, the objectives of this paper were to perform pretreatment comparison studies with cellulase enzymes and dilute solutions of H_2SO_4, NaOH and $NaHCO_3$ as alternatives to traditional petroleum ether pretreatment, and to interface pretreatment with water extraction.

2. Methods

2.1. Plant material

Milk thistle seed was purchased from Frontier (Norway, IA) and stored at 4 °C. The seed was ground in a household coffee grinder to a particle size of 0.4 mm, as determined by ASAE standard S319.1 (ASAE, 2002). Since size reduction prior to extraction is also a pretreatment technique, smaller particle sizes will likely enhance the rates or even possibly the yields during extraction. Abu Jadayil et al. (1999) presented a proximate analysis of milk thistle seed (three replicates with CV < 5%), and showed that the seed contained 5.8 g moisture, 19.1 g protein, 26.3 g fat, 25.4 g crude fiber, 4.8 g ash and 9.8 g iron per 100 g dry matter. The energy content was 410 kcal per 100 g dry matter.

2.2. Chemicals

Silybinin was purchased from Sigma (St. Louis, MO). Silychristin and silydianin were obtained from Phytolab (Hamburg, Germany). H_2SO_4 was secured from Fisher Scientific (Springfield, NJ), $NaHCO_3$ from Mallinckrodt (Phillipsburg, NJ) and cellulase (Trichoderma longibrachiatum, 0.61 U/mg) from Fluka (Milwaukee, WI). NaOH, methanol and petroleum ether were purchased from EM Science (Darmstadt, Germany), and ethanol was obtained from AAPER (Shelbyville, KY). At the time that this work was conducted, no standards were available for either isosilybinin A or for isosilybinin B.

2.3. Pretreatment studies

For each pretreatment, 8 g of ground milk thistle seed were added to 72 ml of either 1.2% NaOH (w/w), 1.5% H_2SO_4 (w/w), 2% $NaHCO_3$ (w/w), water or a solution of 0.14% (w/w) cellulase. The mixture was placed in 250 ml brown bottles and agitated at 60 rpm for 24 h in a shaking water bath (Precision, Winchester, VA). Depending on the experiment, the temperature was set at 40, 50, 60 or 70 °C. After the 24 h pretreatment, samples were centrifuged at 292*g* for 10 min. The supernatant was decanted and the seed residue was air-dried for 24 h at room temperature. For data presented in Tables 1 and 2, the control for pretreatment consisted of extracting 2 g of ground milk thistle seed in a Soxhlet apparatus with 200 ml of petroleum ether. During the petroleum ether or ethanol Soxhlet extractions,

Table 1
Effect of pretreatment on flavonolignan yields (mg/g seed) obtained from the extraction of *S. marianum* seed meal

Pretreatment	Yield (mg/g seed)				
	SC	SD	SA	SB	Total
Control[A] (petroleum ether)	2.77 ± 0.25^{a}	14.19 ± 1.13^{b}	2.12 ± 0.17^{b}	3.52 ± 0.29^{b}	24.24 (100%)
No pretreatment	2.61 ± 0.07^{a}	12.90 ± 0.06^{b}	2.00 ± 0.02^{b}	3.32 ± 0.04^{b}	20.70 (85%)
1.2% NaOH (w/w)	0.00 ± 0.00^{b}	0.19 ± 0.01^{c}	0.00 ± 0.00^{d}	0.00 ± 0.04^{d}	0.20 (1%)
1.5% H_2SO_4 (w/w)	3.12 ± 0.47^{a}	19.12 ± 3.26^{a}	3.15 ± 0.44^{a}	4.43 ± 0.65^{a}	28.93 (119%)
0.14% Cellulase (w/w)	2.80 ± 0.31^{a}	13.43 ± 1.55^{b}	2.26 ± 0.22^{b}	3.74 ± 0.36^{ab}	22.27 (92%)
2% NaHCO3 (w/w)	0.24 ± 0.07^{b}	0.46 ± 0.06^{c}	0.42 ± 0.11^{c}	0.89 ± 0.25^{c}	2.49 (10%)
Water	2.45 ± 0.09^{a}	11.40 ± 0.62^{b}	2.00 ± 0.09^{b}	3.30 ± 0.15^{b}	18.59 (77%)

Pretreatments were performed at 50 °C for 24 h, followed by extraction in boiling ethanol for 4 h in a Soxhlet apparatus. SC, silychristin; SD, silydianin; SA, silybinin A; and, SB, silybinin B.

Numbers in parentheses indicate the percentage recovered relative to the control.

The superscript letters following the calculated means and standard deviations are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at $p < 0.05$.

[A] Control consisted of successive extractions in a Soxhlet apparatus with petroleum ether and with ethanol.

Table 2
Effect of temperature (40, 50, 60 and 70 °C) for water and 1.5% H_2SO_4 (w/w) pretreatment, followed by a 4 h extraction with ethanol at 60 °C on flavonolignan yield (mg/g seed)

Pretreatment	Yield (mg/g seed)				
	SC	SD	SA	SB	Total
Control[A] (petroleum ether)	4.76 ± 0.80^{a}	11.53 ± 2.36^{abcd}	2.81 ± 0.47^{a}	4.41 ± 0.76^{a}	23.50
No pretreatment	3.47 ± 0.43^{ab}	7.88 ± 1.46^{abcd}	2.13 ± 0.24^{a}	3.31 ± 0.41^{a}	16.78
1.5% H_2SO_4 (w/w), 40 °C	2.72 ± 0.14^{ab}	16.98 ± 1.21^{a}	2.43 ± 0.26^{a}	3.50 ± 0.22^{a}	25.64
1.5% H_2SO_4 (w/w), 50 °C	2.99 ± 0.43^{ab}	16.41 ± 1.32^{a}	2.73 ± 0.39^{a}	3.66 ± 0.33^{a}	25.78
1.5% H_2SO_4 (w/w), 60 °C	2.83 ± 0.71^{ab}	14.74 ± 2.90^{abc}	2.36 ± 0.35^{a}	2.94 ± 0.54^{a}	22.87
1.5% H_2SO_4 (w/w), 70 °C	2.50 ± 0.31^{ab}	16.10 ± 1.77^{ab}	3.18 ± 0.65^{a}	4.39 ± 1.06^{a}	26.17
Water, 40 °C	2.43 ± 0.65^{ab}	5.77 ± 2.29^{cd}	1.95 ± 0.53^{a}	3.28 ± 0.89^{a}	13.43
Water, 50 °C	3.44 ± 2.27^{ab}	8.31 ± 8.01^{abcd}	2.99 ± 2.01^{a}	5.12 ± 3.43^{a}	19.86
Water, 60 °C	1.93 ± 0.27^{b}	6.87 ± 3.34^{bcd}	1.55 ± 0.27^{a}	2.71 ± 0.38^{a}	13.09
Water, 70 °C	2.01 ± 0.57^{b}	2.77 ± 1.44^{d}	1.83 ± 0.55^{a}	3.18 ± 0.94^{a}	9.79

The superscript letters following the calculated means and standard deviations are an indication of similarity in the data. Values sharing a letter within a column are not significantly different at $p < 0.05$.
SC, silychristin; SD, silydianin; SA, silybinin A; and, SB, silybinin B.
[A] Control consisted of successive extractions in a Soxhlet apparatus with petroleum ether and with ethanol.

it was observed that while siphoning the liquid into the round bottom flask, boiling ceased for about a minute, but then quickly resumed. Thus, the average temperature for the Soxhlet experiments was the normal boiling point of the solvents. For all of the tested conditions presented in Table 1, including the control and the 'no pretreatment', the seed meals were subsequently placed in thimbles (Whatman, Maidstone, England), and extracted with 200 ml of 95% ethanol for 4 h in a Soxhlet apparatus. On average, five rinsing cycles per hr were recorded during Soxhlet operation. For all tested conditions presented in Table 2, including the control and the 'no pretreatment', the pretreated seed meals were added to 250 ml brown glass bottles that contained 60 ml of ethanol, and were placed in a 60 °C shaking water bath for 4 h at an agitation rate of 60 rpm, as previously described by Wallace et al. (2005). All pretreatments and extractions were done in triplicate. From each of the pretreatments and extractions, one ml aliquots were sampled and evaporated to dryness under a stream of nitrogen at room temperature. To the dried aliquots, one ml of methanol was added, and the resulting solution was then vortexed and filtered (0.45 μm Spartan-13, Schleicher and Schuell, Keene, NH). The samples were analyzed by HPLC as described below.

2.4. Hot water extraction

In a method that was similar to the pretreatment studies, 8 g of milk thistle seed were pretreated in 1.5% H_2SO_4 (w/w), at 60 rpm, 50 °C for 18 h in 250 ml brown bottles. The solids–liquid ratio was maintained at 10% (w/v). After the pretreatment, the mixture was centrifuged and the resulting seed meal solids were air-dried. Two grams of pretreated seed meal were placed in a thimble in a Soxhlet apparatus with 200 ml of boiling water for 4 h. All extractions were done in triplicate. The control consisted of 8 g of seed extracted in a Soxhlet apparatus with 200 ml of petroleum ether, of which 2 g of seed meal were subsequently extracted with 200 ml of 95% ethanol, also in a Soxhlet apparatus. For all extractions, aliquots of 1 ml were sampled, evaporated, reconstituted in 1 ml of methanol and analyzed by HPLC as described below.

2.5. HPLC analysis

The silymarins were quantified by HPLC analysis as described by Wallace et al. (2003a) using a Waters system (Milford, MA), composed of an Alliance 2690 separations Module and a 996 photodiode array with a Symmetry® (Waters, Milford, MA, USA) C_{18} pre-column and C_{18} column (150 mm × 4.6 mm, 5 μm) maintained at 40 °C. The flavonolignan separation was obtained with a water/methanol gradient system, flowing at 0.75 mL/min. A 10 μL sample volume was injected. Detection was at 290 nm. Flavonolignans calibration curves were generated at concentrations between 1.000 and 0.065 mg/ml.

2.6. Statistical analysis

Statistical analyses were performed with JMP software (SAS Institute Cary, SC) using one way ANOVA with Tukey analysis set at $p < 0.05$.

3. Results

3.1. Initial pretreatment screening

Table 1 presents the flavonolignan yields resulting from Soxhlet ethanol extraction preceded by pretreatments with 1.2% NaOH (w/w), 2% $NaHCO_3$, 1.5% H_2SO_4, 0.14% cellulase and water. The NaOH, $NaHCO_3$, H_2SO_4, cellulase and water pretreatments decreased the initial mass of seed (oil removal) by 21%, 11%, 9%, 12% and 6%, respectively, indicating that the water pretreatment removed the least amount of oil. As a control, milk thistle seeds were successively extracted in a Soxhlet apparatus with

Table 3
Comparison of flavonolignan yields between petroleum ether/ethanol Soxhlet extractions and H_2SO_4 pretreatment followed by water Soxhlet extraction

Pretreatment	Yield (mg/g seed)				
	SC	SD	SA	SB	Total
Petroleum ether and ethanol Soxhlet extractions	2.38 ± 0.17	11.29 ± 0.60	1.66 ± 0.13	2.95 ± 0.22	18.28
1.5% H_2SO_4 (w/w) pretreatment followed by water Soxhlet extraction	3.17 ± 0.11	17.67 ± 0.86	1.63 ± 0.04	2.66 ± 0.08	25.13

petroleum ether and with ethanol. Pretreatment with petroleum ether decreased the mass of seed by 25%, such that the pretreatments in this study are not as effective, if oil removal is the major goal of pretreatment. When considering the total flavonolignan yield, petroleum ether pretreatment followed by Soxhlet ethanol extraction resulted in a 17% increase of total flavonolignan yield in comparison to directly extracting the meal in ethanol; however, the differences in individual flavonolignan concentrations were not significantly different. NaOH and $NaHCO_3$ pretreatments resulted in the lowest yields in individual flavonolignan concentrations and in total yields. This is not surprising since pretreatment with alkali will likely dissolve the flavonolignans, and should be avoided. A cellulase pretreatment resulted in a 20% increase in total flavonolignan yield; however, with the exception of SB, the differences in individual flavonolignan concentrations were not statistically different. When compared to the control, the use of 1.5% H_2SO_4 as a pretreatment resulted in the highest flavonolignan concentrations and total flavonolignan yields. Thus, the H_2SO_4 pretreatment was considered a viable alternative to petroleum ether. NaOH and $NaHCO_3$ as pretreatments were discarded because of the low flavonolignan yields. Although water pretreatment resulted in flavonolignan yields that were 23% lower than those obtained with petroleum ether, this pretreatment option was retained for further examination because it presented a "green" alternative to acid pretreatment. Pretreatment with enzymes was not further explored because of the high costs associated with enzyme use. However, significant advances in cellulase effectiveness and cost have occurred in the past few years by companies such as Genencor International and Novozymes (US Department of Energy, 2007). Both companies have reported more than a 10-fold decrease in the cost of enzymes. With continued work, cellulase costs of $0.10 per gallon of ethanol or less, the cost target established by the US Department of Energy Biomass Program, are expected. These advances should help to make enzyme use for cellulose conversion, as well as extraction pretreatment, technically and economically feasible.

3.2. Temperature effects on selected pretreatments

All of the pretreatments that were presented in Table 1 were conducted at 50 °C. Table 2 presents individual and total flavonolignan yields obtained from water and 1.5% H_2SO_4 (w/w) pretreatments at 40, 50, 60 and 70 °C followed by extraction with ethanol at 60 °C for 4 h in a shaker bath. The control consisted of a petroleum ether extraction in a Soxhlet apparatus, followed by an ethanol extraction in a 250 ml bottle. When compared to the control, direct extraction without pretreatment resulted in a 40% reduction in total flavonolignans. With the exception of water as a pretreatment at 60 and 70 °C, the resulting concentrations of SC, SA and SB were not significantly different. However, the yields of SD were statistically different when the seed meals were treated with H_2SO_4 or with water. When pretreated with H_2SO_4, the concentrations of SD were at least 104% higher than when pretreated with water. Of the extraction solvents and temperatures tested, pretreatment with H_2SO_4 at 40 and 50 °C yielded the highest concentrations of SD.

3.3. Effects of pretreatment time on selected pretreatment techniques

Experiments carried out to assemble the results presented in Tables 1 and 2 exhausted the milk thistle seed supply. Further experiments were pursued with a new seed supply, which was lower by 19%, 18%, 20% and 15% in SC, SD, SA and SB, respectively. With this new seed supply in hand, pretreatment duration (6, 12, 18 and 24 h) with petroleum ether was once again investigated. The yields of SA were 2.71 ± 0.09, 2.12 ± 0.05, 3.89 ± 0.30 and 3.06 ± 0.20 for the 6, 12, 18 and 24 h pretreatments, respectively. Similar results were also obtained for SC, SD and SB for both water and H_2SO_4 pretreatments. For convenience, further experiments were conducted using an 18 h pretreatment time.

3.4. Combining H_2SO_4 pretreatment with water extraction time

The pretreatment tests established that pretreating the milk thistle seed with 1.5% H_2SO_4 (w/w) at 50 °C for 18 h could replace the classical petroleum ether pretreatment. Duan et al. (2003) showed that it was possible to extract flavonolignans with water. Table 3 shows the individual and total flavonolignan yields obtained when combining the H_2SO_4 pretreatment with water extraction. A comparison between the classical petroleum ether/ethanol extraction and the H_2SO_4/water extraction is also shown in Table 3. With the exception of SD, the yields obtained with both treatments were similar.

4. Discussion

It was necessary to obtain a second supply of milk thistle seed, which proved to have a lower concentration of flav-

onolignans. Variation in phytochemical content is typical of natural products, as the secondary metabolite content varies from batch to batch, often because of agronomic reasons (Tanko et al., 2005). Different flavonolignan contents of seed batches have been previously reported (Carrier et al., 2002; Wallace et al., 2003a; Benthin et al., 1999). A different flavonolignan content of the seed material results in varying flavonolignan contents in finished products. Davis-Searles et al. (2005) analyzed the flavonolignan content of four commercial sources of silymarin and reported nearly a 20-fold difference in SD content.

Replacing organic extraction solvents with aqueous preparations is desirable because of the environmentally friendly nature of water, and also because aqueous preparations can be better interfaced with energy conversion unit operations, such as the pretreatment of cellulosic material for ethanol production. The treatments presented in Table 1 were selected because of literature precedents. Li et al. (2001) showed increased saccharification of corn stover after pretreatment with 0.3 N (1.2% (w/w)) NaOH. Ammonia pretreatment of agricultural residues was examined by Belkacemi et al. (1998). Allen et al. (2001), working on poplar sawdust, and Kim et al. (2002), working on wood chips, both reported that pretreating biomass with 1.2–1.5% H_2SO_4 (w/w) increased ethanol yields. Pretreatment with enzymes, such as cellulase, increased the lutein concentration of extracts from marigold flowers (Barzana et al., 2002). Although commonly used as a biomass pretreatment in the biofuels industry, the results presented in Table 1 showed that pretreating the milk thistle seed meal with basic solutions was to be avoided. Compared to the control, the use of enzymes did not increase the flavonolignan content, and were not pursued as a viable pretreatment option. The use of water, on the other hand, resulted in flavonolignan concentrations similar to those obtained with direct ethanol extraction. Because of its simplicity and the fact that water is a ''green'' solvent, water was thus pursued as a pretreatment option. In comparing water pretreatment at 40, 50, 60 and 70 °C, SA and SB concentrations were not affected by the water temperature; however, the SD concentrations increased by 200%. H_2SO_4 pretreatment resulted in an increase in total silymarin concentration, mainly because of the increase in SD yields.

Previous studies (Alvarez Barreto et al., 2003; Duan et al., 2003; Wallace et al., 2003b) showed that it was possible to extract flavonolignans with water. The results presented herein indicate that it is possible to replace petroleum ether/ethanol with H_2SO_4/water for the extraction of flavonolignans from milk thistle seed meal. The H_2SO_4/water extraction resulted in an increase of SD over the petroleum ether/ethanol extraction. Of the preparations tested, Davis-Searles et al. (2005) reported that SD alone was not the most active flavonolignan in inhibiting the growth of three prostate cancer cell lines. In fact, the most active flavonolignan in suppressing the growth of the cancer cells was ISB (Davis-Searles et al., 2005). Unfortunately, at the time that this present work was conducted, ISA and ISB were not available as reference compounds, and henceforth, were not quantified. Aside from flavonolignan extraction, hot water may facilitate the extraction of lipids and polyphenols from milk thistle seed meal, rendering the preparation more bioavailable. Of the pretreatments tested in Table 1, water decreased the biomass content by the lowest amount, which points to the fact that oils must have remained in the biomass, possibly contributing to better bioavailability of the flavonolignans. It should be remembered that anecdotal uses of milk thistle were from seed extracts as a tea preparation and not as an ethanol extract. In future work, the oil content of the water extract will be measured. Unfortunately, extraction of phytochemicals with water does have some disadvantages, namely in downstream separation processes. Concentration of the phytochemical stream requires more energy than that of hydrocarbon solvents. Therefore, a cost comparison between solvent and water extraction should be conducted in terms of their respective recovery strategies. Thus, future work should examine the scale-up of such process, specifically examining the costs associated with handling water in terms of boiling and evaporation.

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