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Extraction of Nutraceuticals from Milk Thistle

Part II. Extraction with Organic Solvents

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Abstract

Seeds from milk thistle (*Silybum marianum* Gaert L.) contain flavanolignan and dihydroflavanol compounds that have interesting and important therapeutic activities. The recovery of these silymarin compounds generally involves a two-step defatting and extraction process using organic solvents. This study examined the batch, single-stage extraction of whole and defatted seeds using ethanol, methanol, acetonitrile, and acetone as the solvents. In extracting defatted milk thistle seeds with organic solvents, extraction with ethanol resulted in the highest silymarin yield, although some potential degradation was observed. The maximum yields of taxifolin, silychristin, silydianin, silybinin A, and silybinin B in ethanol were 0.6, 4.0, 0.4, 4.0, and 7.0 mg/g of defatted seed, respectively. However, if silybinin A were the diastereoisomer of choice, methanol would be the preferred extraction solvent because it yielded the highest silybinin A to silybinin B ratio. Interestingly, lipid removal is an important extraction step, because defatted material yields twice the silymarin concentration.

Index Entries: Milk thistle; extraction; ethanol; methanol; silymarin; acetone; acetonitrile.

Introduction

Seeds from milk thistle (*Silybum marianum* Gaert L.) contain flavanolignan and dihydroflavanol compounds that have interesting and important therapeutic activities. Milk thistle flavanolignan and dihydroflavanol

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are also referred to as silymarin. The flavanolignan silybinin shows potential for reducing bilary cholesterol concentrations *(1)*, and in the intervention of hormone refractory human prostate cancer *(2)*. In addition, combinations of the flavanolignans silybinin and silychristin have been shown to decrease the nephritic effects of chemical injury *(3)*. Hence, there is substantial evidence that indicates the therapeutic benefits of silymarin. Interestingly, silymarin products are sold under different categories in Europe than in North America. American milk thistle products are sold as dietary supplements in a $45,000,000 industry *(4)*, while the European Union regulates them as therapeutic products *(1)*.

With more North Americans using botanical preparations, there is growing concern about their safety and efficacy. A quick analysis of American off-the-shelf milk thistle products showed that the labels and the actual content often do not match, to the point that one product was actually devoid of flavanolignans *(5)*. Similar testing of off-the-shelf gingko *(6)* and ephedra *(7)* products also yielded inconsistencies between product content and product label. Botanicals fall under the dietary supplements umbrella, and, unfortunately, the corresponding products vary much more than what is allowed by the pharmaceutical manufacturers. However, as clinical evidence demonstrates the efficacy of specific compounds, standardization, potency, safety, and dose response will become important parameters, requiring extensive documentation.

The production of milk thistle products involves a two-step extraction process. Before being extracted for their flavanolignan content, milk thistle seeds must first be defatted to remove the ~25% lipid content *(5,8)*. However, the open scientific literature on milk thistle extraction is scarce. The Deutsches Arzneibuch procedure for flavanolignan extraction stipulates that the seeds must first be extracted in a petrol Soxhlet for 4 h, followed by a methanol Soxhlet for 5 h *(9)*. The work of Benthin et al. *(9)* reported on the adaptation of the Deutsches Arzneibuch procedure to pressurized liquid extraction, using petrol and methanol as the extraction solvents. A procedure in which the seeds were frozen, ground, and defatted with hexane and then extracted with acetonitrile for maximal flavanolignan recovery has also been reported *(10)*. Thus, industrial extraction procedures imply the use of organic solvents such as petrol, methanol, and acetonitrile for flavanolignan extraction. Although specific solvents are proposed, information on liquid-to-solvent ratios, extraction temperatures, and extraction rates is scarce. To increase the quality of products and the efficiency of extraction, the extraction step should be well characterized, in terms of both extraction conditions and appropriate solvents.

The purpose of this study was to investigate flavanolignan extraction (silymarin) from milk thistle seeds using ethanol, methanol, acetonitrile, and acetone as the extraction solvents. Batch extraction data were obtained at the normal boiling points of the solvents as a function of time. The extraction of whole and defatted seed was also compared.

Materials and Methods

Extraction Experiments

Milk thistle seeds were purchased from Frontier Herbs (Norway, IA) and ground with a coffee grinder to an average particle size of 0.4 mm. Extraction experiments were conducted at the normal boiling point of the respective solvent (methanol, ethanol, acetonitrile, or acetone). For all experiments, 2 g of seed (contained in a cheesecloth bag) was extracted in 200 mL of the corresponding solvent. The boiling flask was heated in an electric mantel, and water was used to condense the vapor.

Extraction samples were taken in triplicate every 30 min, including time zero, using a 1 mL pipet. Time zero was set as the time when the solvent started boiling. The aliquots were placed in preweighed test tubes and weighed to determine aliquot weight. Subsequently, the aliquots were evaporated to dryness under a stream of nitrogen. To the dried sample, 1 mL of methanol was added, after which the solution was vortexed and centrifuged (10*g*). The supernatant was filtered and analyzed, as described next.

Chemical Analysis

Silymarin concentrations were determined by high-performance liquid chromatography (HPLC) using a Waters system (Milford, MA) composed of an Alliance 2690 separations module and a 996 Photodiode Array, controlled with Millennium^{32} chromatography software. Silymarin compounds were separated using a Symmetry® (Waters) C_{18} precolumn placed in series with a Symmetry (Waters) C_{18} column (150 × 4.6 mm, 5 µm), both at 40°C. A 10-µL sample volume was injected. Solvent A was 20:80 methanol:water, while solvent B consisted of 80:20 methanol:water. The gradient program was initiated with 85:15 solvent A:solvent B flowing for 5 min, followed by a linear gradient of 45:55 solvent A:solvent B for 15 min. The proportions of 45:55 solvent A:solvent B were then held constant for 20 min and brought back to 85:15 solvent A:solvent B over 10 min. The flow rate was 0.75 µL/min, and the silymarin compounds were monitored at 290 nm. Peak identification was confirmed by mass spectrometry (Pharmalytics, Saskatoon, Saskatchewan, Canada). Calibration curves were prepared with silybinin from Sigma (St. Louis, MO), taxifolin from Extrasynthese (Lyon, France), and silychristin and silydianin from PhytoLab (Hamburg, Germany). No standard was available for isosilybinin, and thus this compound was excluded from the analysis. The silybinin standard obtained from Sigma contained two distinct peaks, which are further referred to as silybinin A (first peak) and silybinin B (second peak). Sample chromatograms from the extraction of whole and defatted milk thistle seeds are shown in Fig. 1, in which the HPLC procedure was previously described *(5)*.

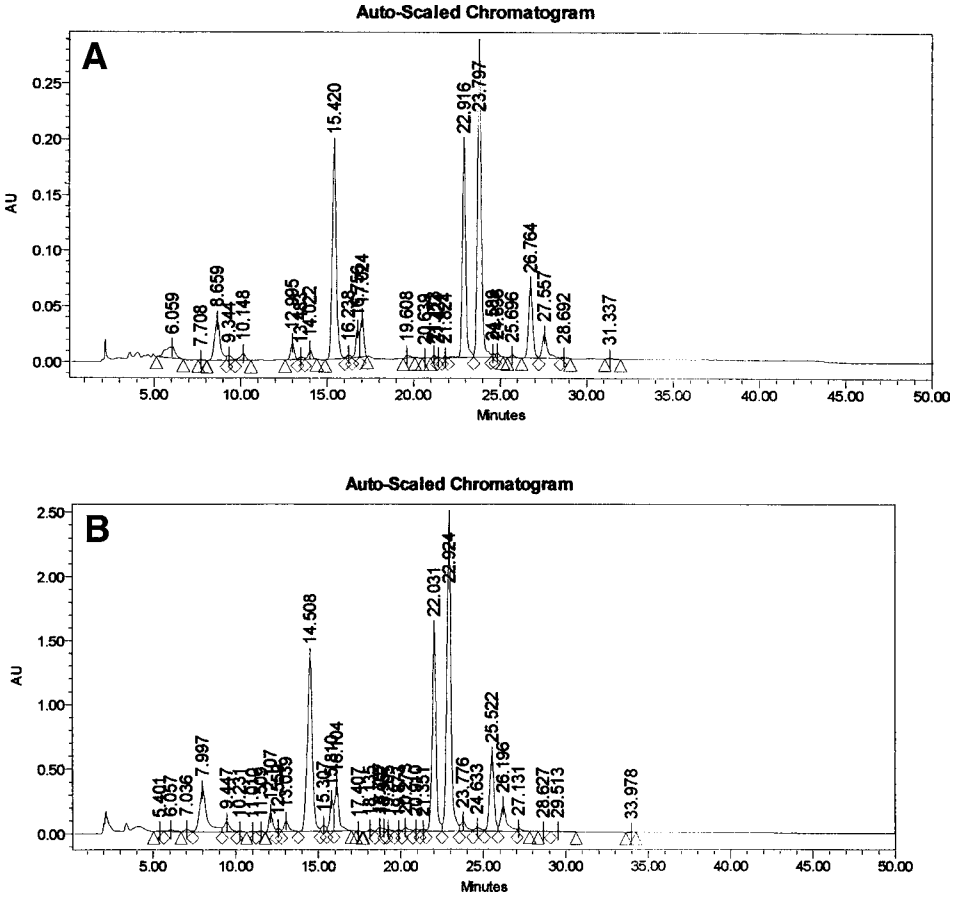

Fig. 1. Typical chromatogram of milk thistle seed extract: **(A)** whole seeds; **(B)** defatted seeds. Retention times of taxifolin, silychristin, silydianin, silybinin A, and silybinin B were 8.7, 15.4, 17.6, 22.9, and 23.8 min, respectively. Note that this particular seed lot contained miniscule amounts of silydianin.

Results and Discussion

Extraction Reproducibility

Experiments were performed to illustrate the similarities and differences in extracting whole and defatted seeds with various organic solvents at their normal boiling points. Figure 2 demonstrates the reproducibility of the concentration-time data in each solvent by showing the silybinin B yields in the extract solvent as a function of time. Three distinct experiments were conducted with each solvent, and three samples were taken at each time point for a total of nine samples per time point. The reproducibility of the data for each of the extracted compounds was generally quite good, with only a few outliers observed in the data trend.

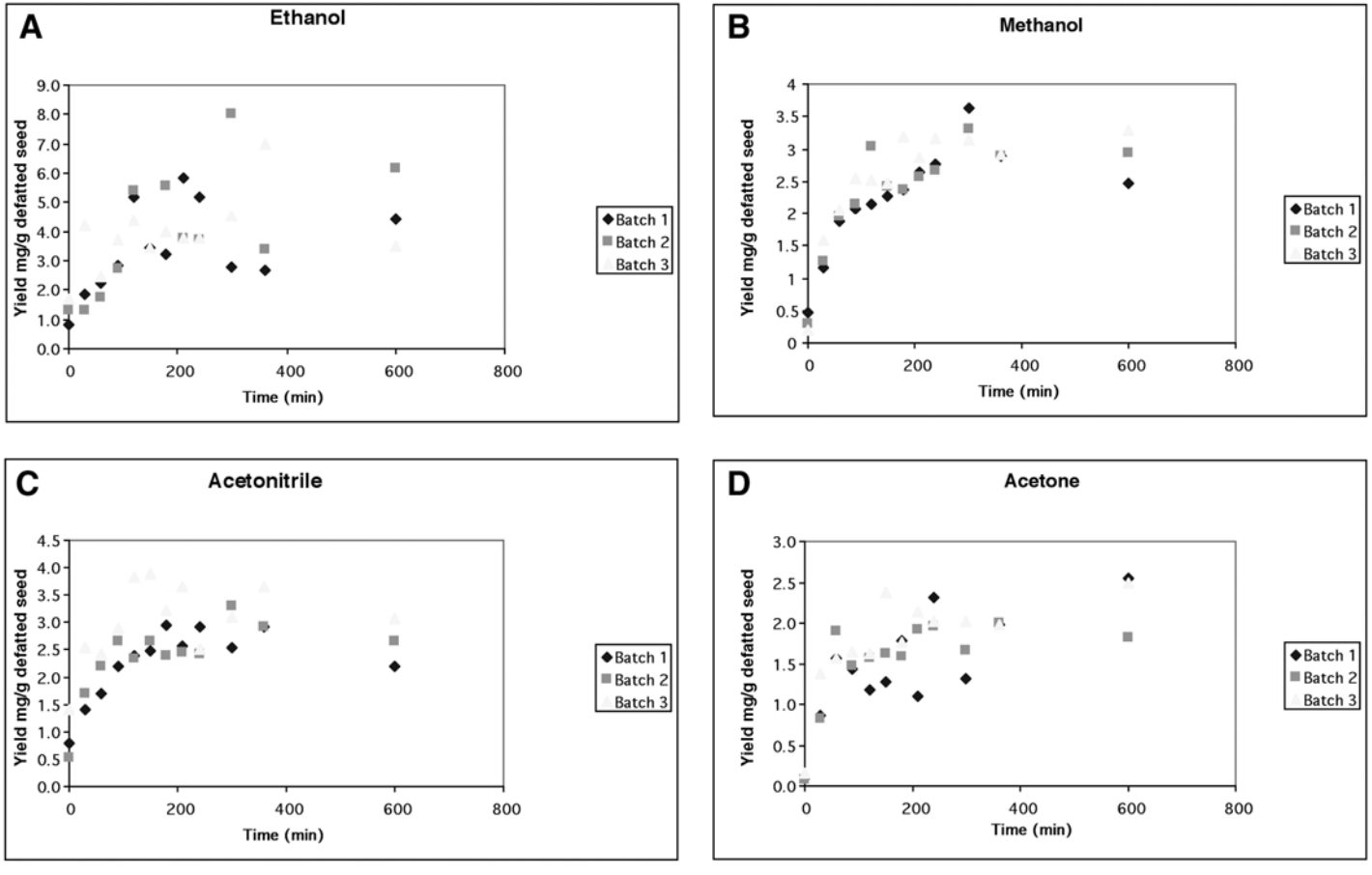

Fig. 2. Silybinin B yield (mg of compound/g of defatted seed) as function of time in ethanol, methanol, acetone, and acetonitrile at their normal boiling points. Results show all the batches for all of the solvents. **(A)** Ethanol; **(B)** methanol; **(C)** acetonitrile; **(D)** acetone.

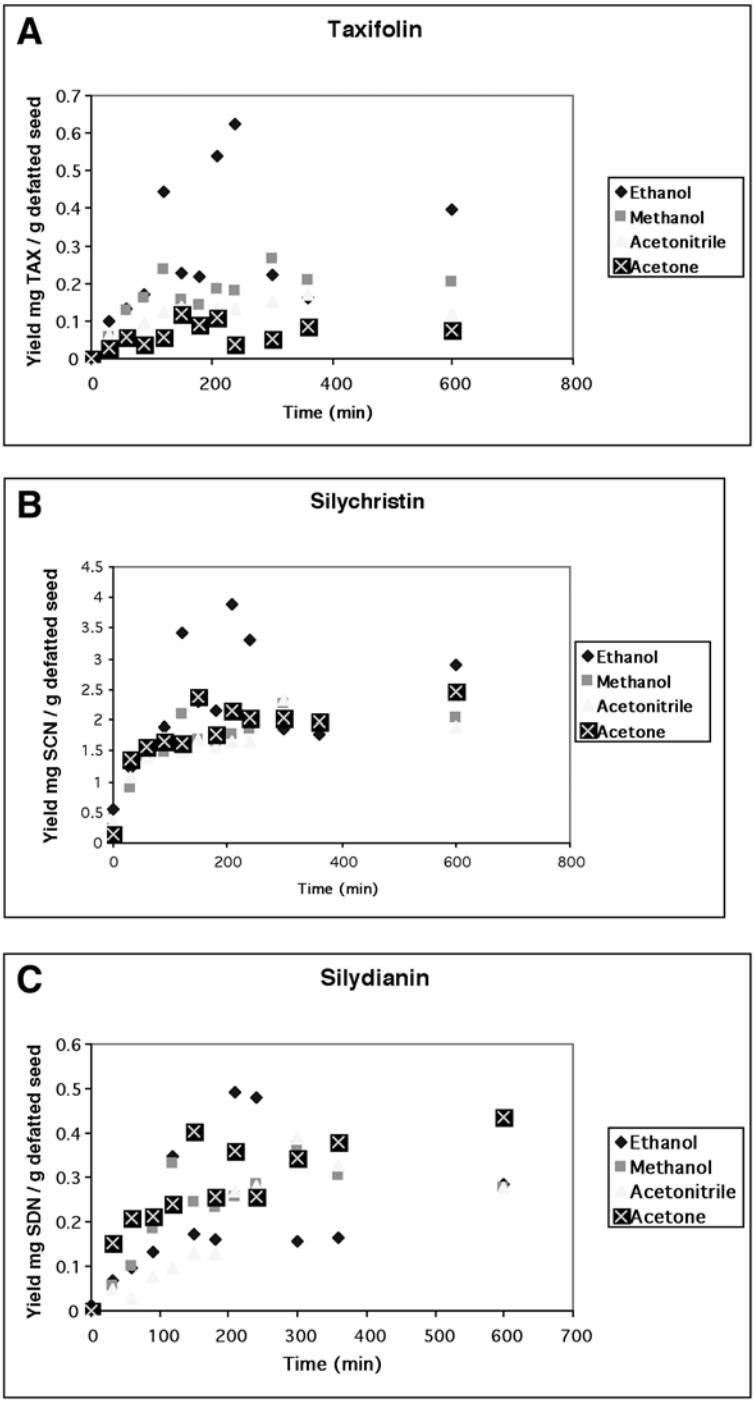

Fig. 3. Silymarin yields (mg of compound / g of defatted seed) as function of time in ethanol, methanol, acetone, and acetonitrile at their normal boiling points. **(A)** Taxifolin (TAX); **(B)** silychristin (SCN); **(C)** silydianin (SDN). *Continued on next page.*

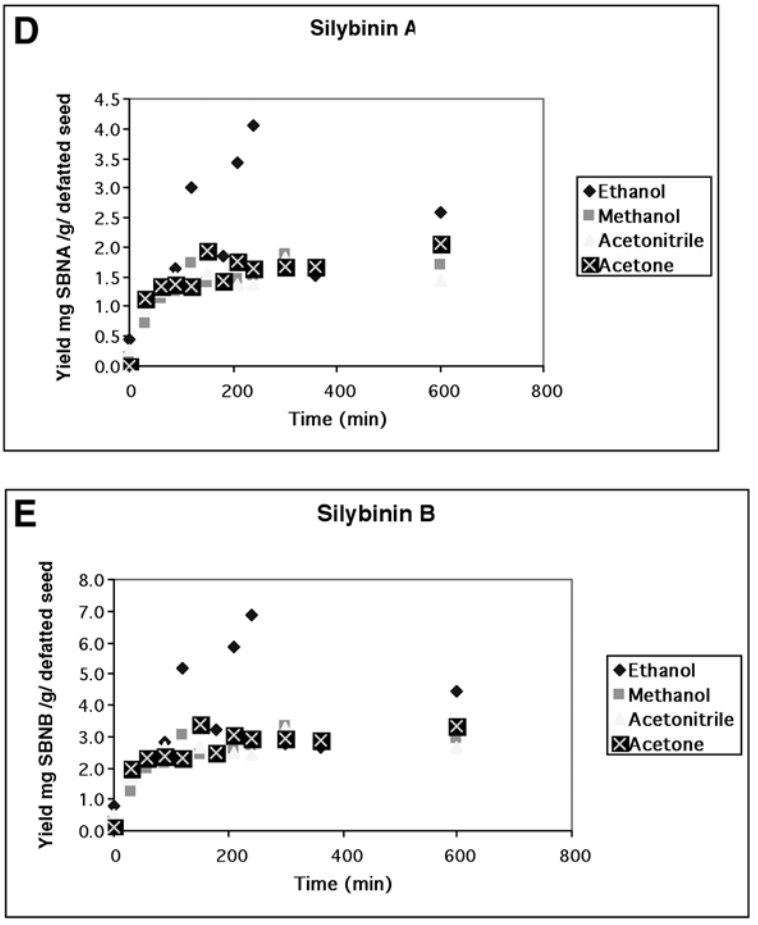

Fig. 3. *Continued from previous page.* **(D)** silybinin A (SBNA); **(E)** silybinin B (SBNB).

Extraction with Organic Solvents

Figure 3 shows typical results from the extraction of taxifolin, silychristin, silydianin, silybinin A, and silybinin B, as the yield of each compound as a function of time in each solvent. As expected, the extraction of each of the silymarins showed a general increase in yield with time, regardless of solvent, as equilibrium between the extracted compounds and the solvent was approached. Some potential degradation of silymarins was observed when extracting with ethanol (as noted by the decrease in yield at long extraction times), a trend that was not observed when extracting with the other solvents. In comparing the yields of the individual silymarins, it is observed that ethanol was clearly the preferred solvent for extraction, followed closely in order by methanol, acetonitrile, and acetone.

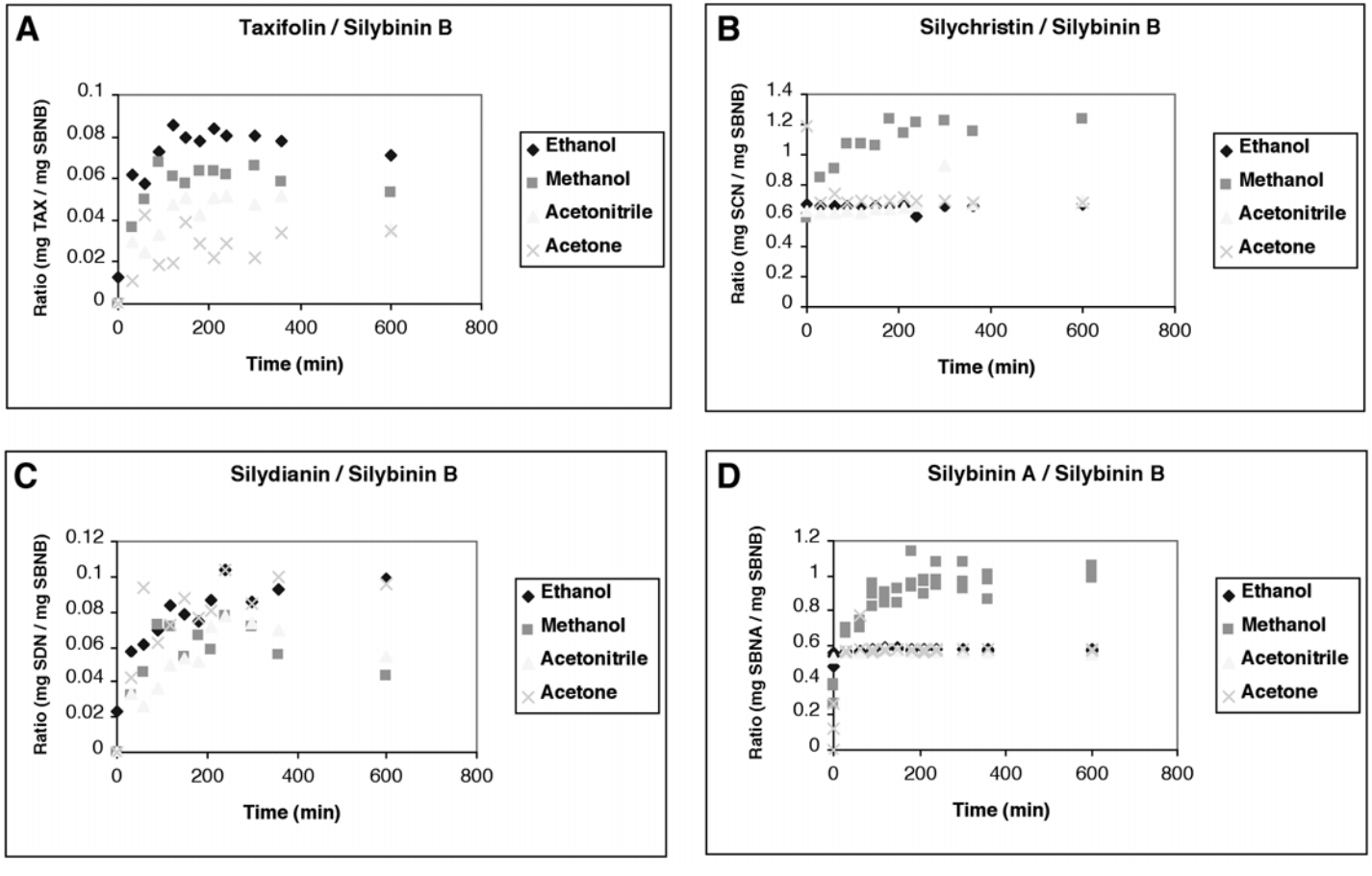

Fig. 4. Compound ratio as function of time in ethanol, methanol, acetone, and acetonitrile at their normal boiling points. **(A)** Taxifolin to silybinin B ratio; **(B)** silychristin to silybinin B ratio; **(C)** silydianin to silybinin B ratio; **(D)** silybinin A to silybinin B ratio.

The maximum yield of taxifolin extracted with ethanol as the solvent was 0.6 mg/g of defatted seed, while the maximum yields of taxifolin with methanol, acetonitrile, and acetone were 0.3, 0.2, and 0.1 mg/g of seed, respectively. The maximum yields of silychristin were 4.0, 2.1, 1.6, and 2.5 mg/g of defatted seed in ethanol, methanol, acetonitrile, and acetone, respectively. In comparing these yields with the yields obtained in a 4-h Soxhlet extraction with ethanol on defatted seeds (0.22 mg of taxifolin and 1.40 mg of silychristin/g of defatted seed), it is seen that the yields of taxifolin and silychristin actually exceeded the yields in the 4-h Soxhlet extraction, which further illustrates the potential for silymarin degradation. The maximum yields of silydianin in the solvents were all about 0.4 mg/g of defatted seed (23% of the Soxhlet results), while the maximum yields of silybinin A in ethanol, methanol, acetonitrile, and acetone were 4.0 (49%), 1.9 (24%), 1.7 (21%), and 2.0 (25%) mg/g of defatted seed, respectively. Finally, the maximum yields of silybinin B in ethanol, methanol, acetonitrile, and acetone were 7.0 (52%), 3.2 (24%), 3.1 (23%), and 3.2 (24%) mg/g of defatted seed, respectively. The yields of silymarins extracted in ethanol in the present work compare well with the results of pressurized liquid extraction *(9)*.

It is interesting to compare the ratios of the extracted compounds in the various solvents. Figure 4 shows the ratios of taxifolin and of each of the silymarins to the quantity of silybinin B, extracted from defatted seeds, as a function of time for each of the solvents at their normal boiling points. Excluding the first few minutes of extraction, the ratios of taxifolin to silybinin B for acetone, acetonitrile, methanol, and ethanol remained relatively constant at 0.02, 0.05, 0.06, and 0.08 mg/mg, respectively. The ratios of silychristin to silybinin B remained constant at about 0.7 mg/mg for ethanol, acetonitrile, and acetone and gradually increased from 0.8 mg/mg and then held at 1.2 mg/mg for methanol. Likewise, the ratios of silybinin A to silybinin B held constant at 0.6 mg/mg for ethanol, acetonitrile, and acetone, while increasing slowly from 0.6 to 1.0 mg/mg for methanol. The ratio of silydianin to silybinin B varied from 0.02 to 0.1 mg/mg for the solvents but generally held in the range of 0.08 mg/mg.

There are three possible rate-controlling steps in the extraction of compounds from a solid matrix: (1) overcoming an energy barrier such as the passage through a cell wall or desorption from a surface, (2) transport through the matrix by diffusion through the bulk material or its pores, or (3) removal by partitioning into the solvent. Basile et al. *(11)* showed that the controlling mechanism for the extraction of monoterpenes, sesquiterpenes, waxes, and lipids from rosemary using superheated water was the partitioning of the compounds into the solvent. They also found a rough correlation between the solubility of the compounds in the solvent and the extraction rates. Goto et al. *(12)* noted that compounds that have similar structures are extracted similarly. Each of the silymarin compounds in the present study are similar in structure and in their solubilities in the extracting solvent. Thus, similar extraction rates should be observed, which is indeed the case.

Table 1
Ratios of Taxifolin, Silychristin, Silydianin, and Silybinin A to Silybinin B in Ethanol, Methanol, Acetonitrile and Acetone[a]

Solvent	Taxifolin/ silybinin B	Silychristin/ silybinin B	Silydianin/ silybinin B	Silybinin A/ silybinin B
Ethanol (defatted seeds)	0.09	0.48	0.07	0.59
Ethanol, (whole seeds)	0.04	0.73	0.02	0.56
Methanol (defatted seeds)	0.06	1.20	0.08	1.00
Acetonitrile (defatted seeds)	0.05	0.06	0.08	0.60
Acetone (defatted seeds)	0.03	0.60	0.10	0.60

[a]Ratios were calculated at time 240 min. Ethanol extractions were conducted on defatted and whole seeds.

A clearer view of the ratios of the extracted compounds can be seen in Table 1, where the ratios are shown after an extraction time of 240 min, a time prior to any compound decomposition. The ratios are nearly constant except for two notable exceptions: methanol extraction shows significantly higher silychristin/silybinin B and silybinin A/silybinin B ratios in comparison with the other solvents. Differences in the taxifolin/silybinin B ratios were also observed but appear to be more closely related to experimental scatter owing to low extract concentrations. These observed differences in the ratios with methanol point toward methanol being a better solvent if silychristin and/or silymarin A are the desired compounds for extraction. This point was apparently also observed by Benthin et al. *(9)*, who recommended methanol as the extraction solvent in pressurized liquid extraction.

Extraction of Whole and Defatted Seeds

Recommended extraction procedures from the literature emphasize the importance of removing the lipids from milk thistle seeds, prior to silymarin extraction. Petrol *(9)* and hexane *(10)* have been suggested; however, no information has been reported on the advantages of a defatting pretreatment step. Hence, extraction experiments were performed on whole and defatted seeds using ethanol as the solvent at its normal boiling point. Figure 1 shows liquid chromatograms for the whole and defatted seeds, obtained under identical extraction conditions. As noted, the chromatogram patterns are identical except for the larger areas of the silymarin compounds obtained while extracting defatted seeds. This result is better illustrated in Fig. 5, where the yields of silymarin compounds (mg/g of equivalent whole seed) are plotted as a function of time for both whole and defatted seeds. In comparing the maximum yields of the silymarins, defatted seeds yielded on average twice the quantity of compounds obtained for whole seeds under identical extraction conditions. The most striking difference was with silydianin (5X), and the least

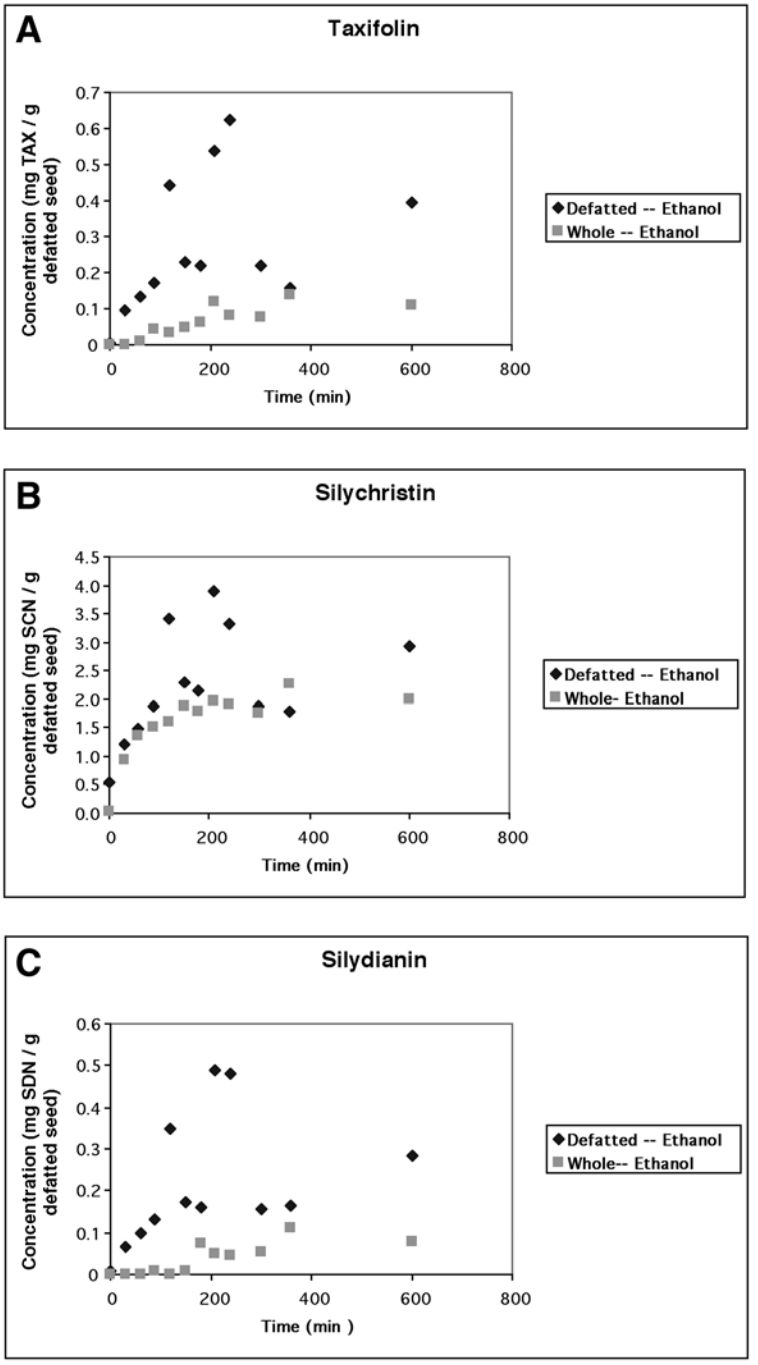

Fig. 5. Comparison between ethanol extraction of whole and defatted milk thistle seeds. Silymarin yields are expressed as milligrams of compound/gram of defatted seed as a function of time. **(A)** taxifolin; **(B)** silychristin; **(C)** silydianin.

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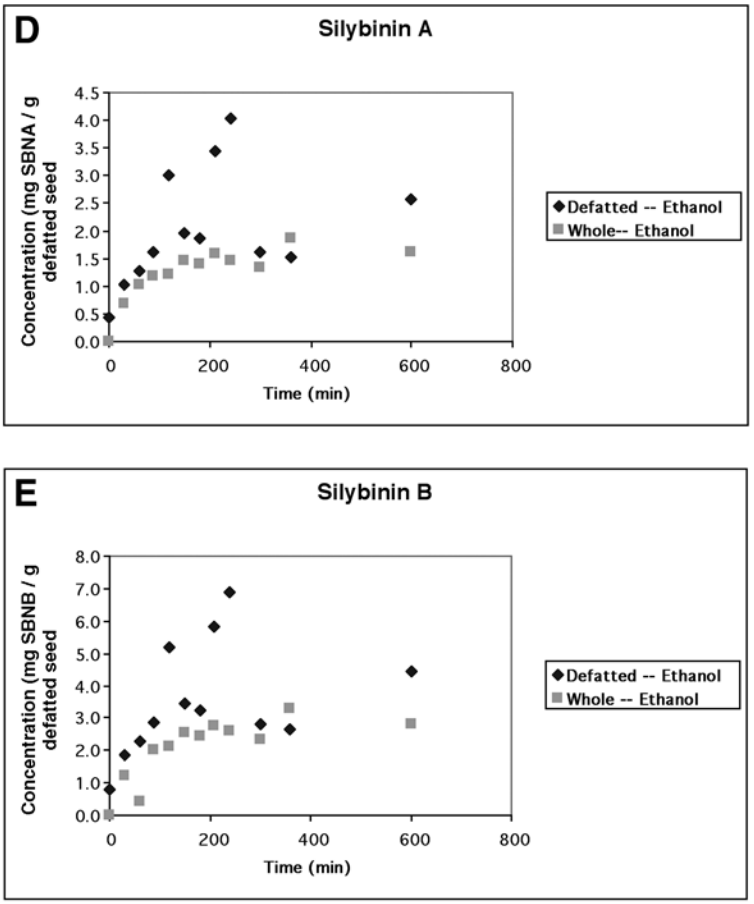

Fig. 5. *Continued from previous page.* Comparison between ethanol extraction of whole and defatted milk thistle seeds. Silymarin yields are expressed as milligrams of compound/gram of defatted seed as a function of time. **(D)** silybinin A; **(E)** silybinin B.

striking was with silychristin. The compound ratios (silymarin compound to silybinin B) in Table 1 for whole and defatted seeds show that only small differences were observed.

In extracting menthone, 1-menthol, menthyl acetate, and other essential oil components from peppermint, Goto et al. *(12)* noted that the extraction rates were slowed because essential oil components must be desorbed from lipid in which the components are absorbed. Fat removal apparently helps to release the compounds from the plant matrix without preferentially affecting the release of the individual compounds. It is possible that pretreatment protocols for fat removal may affect product ratios.

Conclusions

In extracting defatted milk thistle seeds with organic solvents, extraction with ethanol resulted in the highest silymarin yield, although some potential degradation was observed. The maximum yields of taxifolin, silychristin, silydianin, silybinin A, and silybinin B in ethanol were 0.6, 4.0, 0.4, 4.0, and 7.0 mg/g of defatted seed, respectively. However, if silybinin A were the diastereoisomer of choice, methanol would be the preferred extraction solvent because it yielded the highest silybinin A–to–silybinin B ratio. Interestingly, lipid removal is an important extraction step, because defatted material yields twice the silymarin concentration.

Future work in extracting silymarins from milk thistle with organic solvents will focus on temperatures below the normal boiling point. Less compound degradation should occur as the temperature is lowered. Multistage extraction will also be considered in an effort to improve silymarin yields.

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