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# Extraction of silymarin compounds from milk thistle (*Silybum marianum*) seed using hot, liquid water as the solvent

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J.F Alvarez Barreto\*, D.J Carrier<sup>§</sup>, and E.C. Clausen<sup>†</sup>

### ABSTRACT

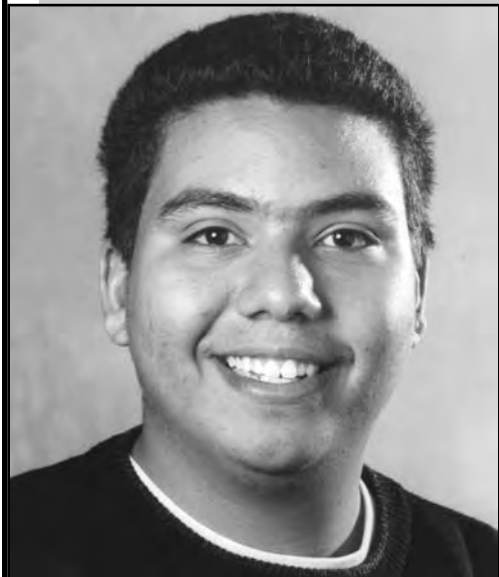
High-value specialty chemicals are usually obtained from natural products by extracting with generally regarded as safe (GRAS) solvents. Because organic solvents are quite often used, high operating and disposal costs occur. When compared to traditional solvents, water is an interesting alternative because of its low operating and disposal costs. Milk thistle contains compounds (taxifolin, silychristin, silydianin, silybinin A, and silybinin B) that display hepatotoxic protection properties. This paper examines the batch extraction of silymarin compounds from milk thistle seed meal in 50°C, 70°C, 85°C and 100°C water as a function of time. For taxifolin, silychristin, silybinin A, and silybinin B, extraction with 100°C water resulted in the highest yields. After 210 min of extraction at 100°C, the yield of taxifolin was 1.2 mg/g of seed while the yields of silychristin, silybinin A, and silybinin B were 5.0, 1.8 and 3.3 mg/g of seed, respectively. The ratios of the extracted compounds, and particularly the ratios at long extraction times, showed that the more polar compounds (taxifolin and silychristin) were preferentially extracted at 85°C, while the less polar silybinin was preferentially extracted at 100°C.

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## MEET THE STUDENT-AUTHOR



José Alvarez

I was born in Cumaná, Venezuela, in front of a beautiful beach. When 4 years old, I moved to the industrial capital of my country, Valencia. I have always been interested in sciences and graduated from high school in this branch. Then I enrolled in chemical engineering, which really was not my favorite area. One year later, I joined a Science Society for Chemical Engineering Students (SOCITEC), where I contributed to the development of many activities related to the area. Three months later, I was elected as the academic coordinator and had to carry out several projects that literally made me fall in love with my career. These projects were mainly in the area of surfactants and catalysis.

In the year 2000, I had the opportunity to transfer to the University of Arkansas. In this institution I participated in interesting research projects in the area of biotechnology, but I always liked catalysis better. Nevertheless, I really enjoyed my work and learned a lot. I graduated in Spring 2002, and now I will start my PhD program in heterogeneous catalysis at the University of Oklahoma, which is an important step to become a researcher. Later in life, I would like to teach in a university in Venezuela.

## INTRODUCTION

Milk thistle (*Silybum marianum*) is an annual or a biennial plant native to the Mediterranean and North Africa. It grows wild throughout Europe, North Africa, the Americas, and Australia, but can also be cultivated (Hamid et al., 1983). The plants can reach a height of 3.1 m with dark shiny leaves and purple to reddish flowers. Milk thistle has an indeterminate growth habit, resulting in staggered flowering and maturity (Carrier et al., 2002). The seeds of the plant contain a group of flavanoid compounds commonly named silymarin (Tittle

and Wagner, 1978).

The dihydroflavonol taxifolin and the flavanolignans (silybinin, isosilybinin, silydianin, and silychristin) are usually encompassed by the term silymarin (Fig 1). Some studies suggest that silybinin reduces the biliary cholesterol concentration (Duke, 1999). It has also been demonstrated that silybinin is useful in the intervention of hormone refractory human prostate cancer (Zi and Agarawal, 1999). Furthermore, the combination of silybinin and silychristin has been found helpful in decreasing the nephritic effects of chemical-induced injury (Sonnenbichler et al., 1999).



Fig. 1. Structures of silychristin (SCN), silydianin (SDN), silybinin (SBN), taxifolin (TXF) and isosilybinin (ISBN).

The Deutsches Arzneibuch (Benthin et al., 1999) procedure for silymarin extraction is a two-step, process in which seeds are first defatted in a Soxhlet extraction with petrol for 4 hrs, followed by a second Soxhlet extraction with methanol for 5 hrs. Using this procedure, reported silybinin yields were 11 mg/g of seed (Benthin et al., 1999). Milk thistle was also extracted using pressurized liquid extraction techniques, in which 12 mg of silybinin/g of seed were obtained (Benthin et al., 1999). In extracting 0.4 mm milk thistle seed meal in a Soxhlet with petrol for 24 hr, followed by an ethanol Soxhlet for 4 hr, Wallace et al. (2002a) reported a silybinin yield of about 16 mg/g of seed meal.

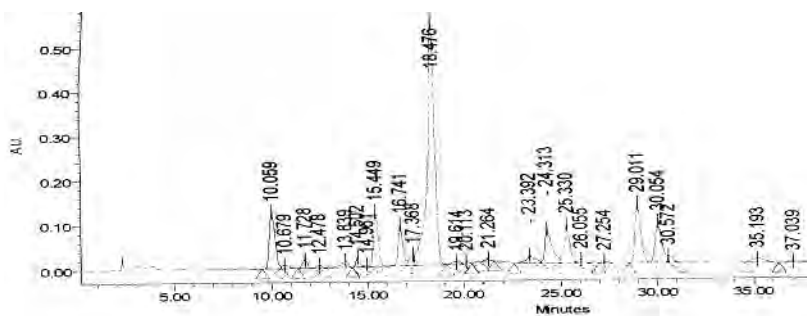
The two-fold difference obtained by Wallace et al. (2002a) over Benthin et al. (1999) may not be significant, since the silybinin content of seed batches varies significantly (Carrier et al., 2002).

Wallace et al. (2002a) reported the analysis of three off-the-shelf milk thistle products, of which only two

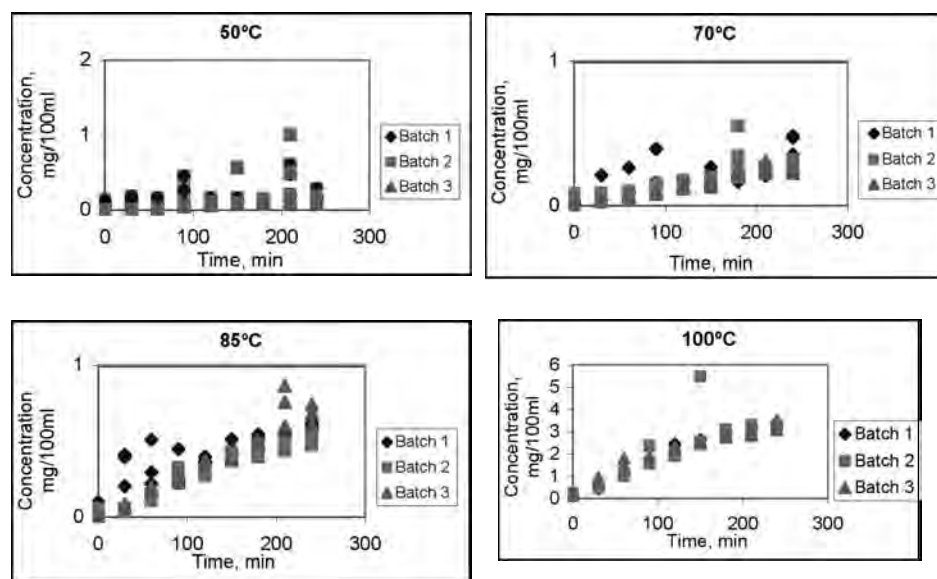
products contained silymarin compounds. Inconsistency between herbal supplement label and product contents is not uncommon. For example, an analysis of ephedra products (Gurley et al., 2000) showed a broad range of ephedra alkaloid content, pointing most likely to manufacturing variation. The lack of consistency among products can be due in part to the extraction step where the desired molecules diffuse from the bulk herb to a solvent phase, usually ethanol, methanol, acetone, hexane, or petroleum ether. To increase the quality of the products, the extraction step should be well characterized, both in terms of rates and appropriate solvents.

The use of hot liquid water as an extraction solvent has recently caught the attention of some researchers (Basile et al., 1998; Kubátová et al., 2001). Water is very useful in extracting polar compounds and may be useful in extracting polar compounds from plant material without prior defatting. In increasing the water temperature

up to its subcritical temperature, a decrease in the dielectric constant is observed. For example, water at 250°C displays a dielectric constant of 27, which is in the realm of that of methanol (33) and ethanol (24). As a result, hot liquid (hot/liquid) water has solubility characteristics, at increased temperature, which are similar to ethanol and methanol. The solubilities of anthracene, pyrene, chrysene, perylene and carbazole (Miller et al., 1998) and of d-limonene, carvone, eugenol, 1,8-cineole and nerol (Miller and Hawthorne, 2000) were determined in 289 K



**Fig. 2.** Typical chromatogram of milk thistle seed extract. Retention times of taxifolin, silychristin, silydianin, silybinin A and silybinin B were 10.59, 18.476, 21.264, 24.313 and 25.330 minutes, respectively. It should be noted that this particular seed lot contained miniscule amounts of silydianin.



**Fig. 3.** Silybinin B concentration in mg of compound per 100 ml as a function of time at different temperatures. Results show all the batches for all temperatures. The maximum time reported is 240 min; experimentally, infinite times corresponded to 300 min for 100°C experiments, and 1020 min for 50°C, 70°C, and 85°C experiments.

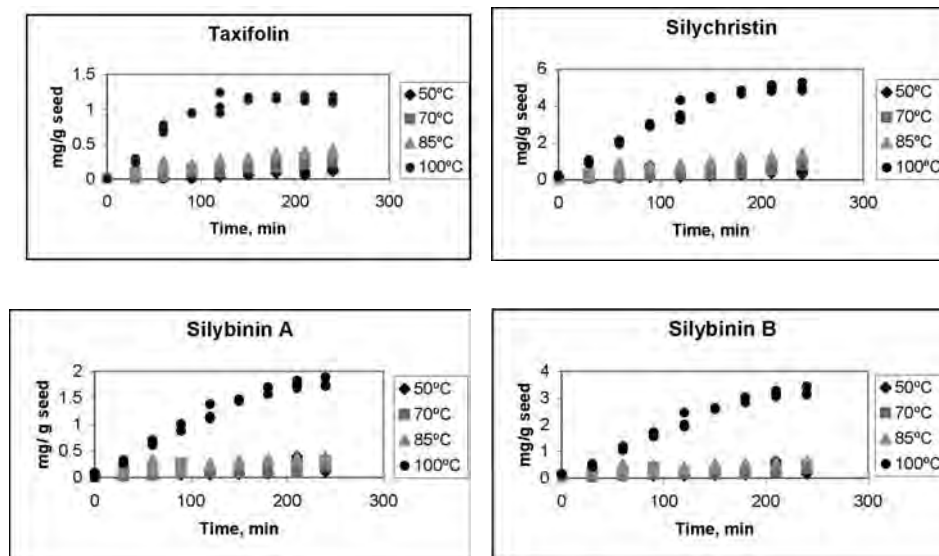
and 498 K (hot/liquid) water, where increases were observed with temperature. Kubátová et al. (2001) showed that the extraction of peppermint compounds using (hot/liquid) water at 175°C required 15 min, as compared to 4 hrs with hydrodistillation. The use of (hot/liquid) water as an extraction solvent shows promise as the search for milder and “greener” solvents is intensifying.

The purpose of this paper is to present results from the extraction of silymarin compounds from milk thistle seeds using (hot/liquid) water as the solvent, a first step in process characterization. Comparisons were made between the compounds extracted as the temperature increased.

## MATERIALS AND METHODS

### Extraction experiments

Milk thistle seeds were purchased from Frontier Herbs (Norway, Iowa) and ground with a coffee grinder to an average particle size of 0.4 mm. Extraction experiments were conducted at 50°C, 70°C, 85°C, and 100°C, using 2 g of seed (contained in a cheesecloth bag) in 200 mL of deionized water. The leaching at 100°C was carried out in a 500 mL glass round-bottom flask, fitted with a condenser for total reflux. The flask was heated in an electric mantel, and water was used to condense the vapor. The leaching experiments at 50°C, 70°C, and 85°C were carried out in 500 mL bottles in a shaker water bath (Dubnoff Metabolic Shaking Incubator, Precision Scientific, Winchester, Virginia) set at 80 strokes / minute. Although the process conditions were slightly different when operating at or below 100°C, the long diffusion times observed in the experiments helped minimize the small differences in the systems.



**Fig. 4.** Concentration in mg of compound per gram of seed as a function of time at different temperatures. results based on the first batch of each temperature. The maximum time reported is 240 min; experimentally, there were infinite times of 300 min for 100°C, and 1205 min for 50°C, 70°C, and 85°C.

Samples of extraction water were taken in triplicate every 30 min, including time 0, using a 1 mL pipette. Time 0 was arbitrarily set as the time when the water started boiling (100°C) or when the temperature of the water in the bottles equilibrated with the set experimental temperature (50°C, 70°C, or 85°C). The aliquots were placed in pre-weighed test tubes and weight determined. Subsequently, the aliquots were evaporated to dryness in a SpeedVac (Savant Instruments, Holbrook, New York). To the dried sample, 1 mL of methanol was added after which they were vortexed and centrifuged (10 g). The supernatant was filtered and analyzed, as described below.

### Chemical Analysis

The silymarin concentrations were determined by HPLC using a Waters system (Milford, Massachusetts) composed of an Alliance 2690 separations module and a 996 Photodiode Array, controlled with Millennium32 chromatography software. Separation of the silymarin compounds was obtained using a Symmetry® (Waters, Milford, Massachusetts) C18 pre-column placed in

**Table 1: Calculated ratio of compound/Silybinin B as a function of temperature. These ratios were calculated at the last sampling point.**

Water Temperature (°C)	Dielectric Constant	Ratio of Compound to Silybinin B (SB)		
		Taxifolin/SB	Silychristin/SB	Silybinin A/SB
50	70	0.916	2.006	0.615
70	64	0.594	1.869	0.639
85	60	0.661	2.237	0.630
100	56	0.352	1.546	0.551

series with a Symmetry® (Waters, Milford, Massachusetts) C18 column (150 mm x 4.6 mm, 5 mm), both at 40°C. A 10 mL 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 mL/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, Missouri), 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 (the first peak) and silybinin B (second peak). A sample chromatogram from the extraction of milk thistle seeds is shown in Fig. 2. The HPLC procedure was previously described by Wallace et al., (2002a).

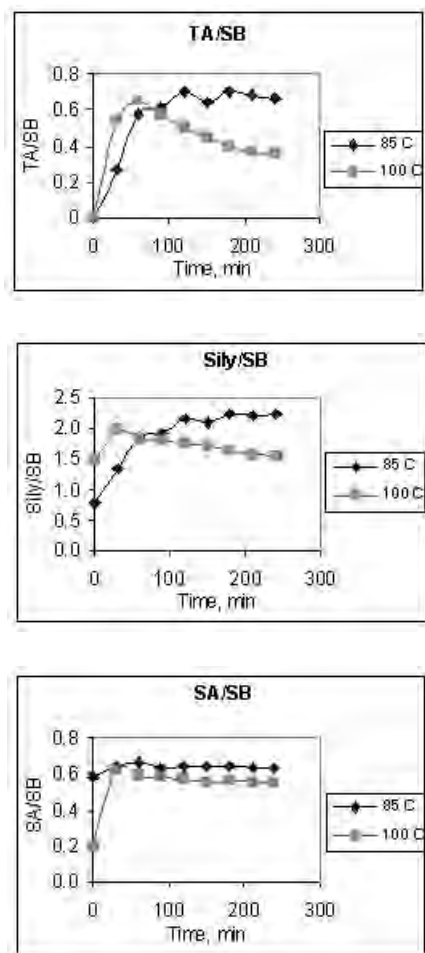
## RESULTS AND DISCUSSION

For all temperatures, three distinct experiments were conducted from which three samples were taken per time point (total of nine samples per time point). Fig. 3 demonstrates the reproducibility of the concentration-time data at each temperature by showing the silybinin B concentration in the extract water with time. The reproducibility of the data improved with increasing temperature as the concentration of the extracted compound increased.

Fig. 4 shows typical results from the extraction of taxifolin, silychristin, silybinin A, and silybinin B presented as the yield of each compound (mg/g of seed) as a function of time and temperature. Each of the extracted compounds showed a consistent pattern of increasing yield with temperature and time. For each of the silymarin compounds, extraction with 100°C water produced the highest yield and concentration of compounds. After 210 min of extraction at 100°C, the yield of taxifolin was 1.2 mg/g of seed while the yields of silychristin, silybinin A, and silybinin B were 5.0, 1.8 and 3.3 mg per g of seed, respectively. After 300 min of extraction, the yields of taxifolin, silychristin, silybinin A, and silybinin B were 0.92, 4.7, 1.8, and 3.4 mg per gram of

seed, respectively (data not shown). A slight decrease in the yield of taxifolin was observed after 150 min, perhaps indicating the onset of decomposition. Water extraction at 100°C yielded about half of the amount of the silybinins obtained in the two-step Soxhlet extraction (with defatting) performed by Wallace et al. (2002a).

The ratios of the concentrations of taxifolin, silychristin, and silybinin A to the concentration of silybinin B at 85 and 100°C as a function of time are shown in Fig. 5. These temperatures were chosen because the flavanolignan concentrations were not as large at temperatures below 85°C. As is noted in Fig 5 (top left), at 85°C the ratio of taxifolin to silybinin B increased rapidly to 0.7/g and then held constant at that level. At 100°C, the



**Fig. 5.** Compound ratio as a function of sampling points for the 85°C and 100°C experiments. Top (A) taxifolin to silybinin B ratio. Middle (B) silychristin to silybinin B ratio. Bottom (C) silybinin A to silybinin B ratio.

ratio reached a maximum of 0.65 g/g and then gradually fell with time to 0.35 g/g. This reduction in the ratio at 100°C shows that the taxifolin concentration reached its maximum faster than silybinin B. A similar behavior for the ratio of silychristin to silybinin B is noted in Fig 5 (top right). At 85°C, the ratio rapidly rose to just above 2.0 g/g, and then gradually increased before leveling out at 2.2 g/g. At 100°C, the ratio increased to a maximum of 2.0 g/g and then gradually fell to 1.5 g/g. The data of Fig 5 (bottom) show that, excluding an initial sharp increase, the ratio of silybinin A to silybinin B at 85°C was constant at 0.65 g/g. At 100°C, the ratio was constant at about 0.6 g/g again excluding the initial sharp period of increase.

These ratios, and particularly the ratios at long extraction times, show that the more polar compounds (taxifolin and silychristin) are preferentially extracted at 85°C while the less polar compounds (silybinin A, and silybinin B) are preferentially extracted at 100°C (see also the data of Table 1). The data reported by Wallace et al. (2002a) showed that the ratios of taxifolin to silybinin B, silychristin to silybinin B, and silybinin A to silybinin B were 0.02, 0.1, and 0.6, respectively. Thus, the ratios of extraction products using water at 100°C more closely resemble the Soxhlet extraction results than the water extractions at temperatures below 85°C. More dramatic differences in polar and nonpolar compound extraction with water are expected as the temperature of liquid water is further increased, thereby lowering the dielectric constant.

Although the yields of taxifolin, silychristin, silybinin A, and silybinin B using water are half of what is reported in ethanol (Wallace et al., 2002a), this technology shows promise because of the omission of the defatting step. An oil removal step was found necessary in the extraction procedures in acetonitrile proposed by Kahol et al. (2001) and in methanol by Benthin et al. (1999). The work of Wallace et al. (2002b) will examine the extraction of defatted milk thistle seed meal using ethanol, methanol, acetonitrile and acetone as the solvent, establishing a platform on which the present water work will be compared to.

Water is not only an interesting alternative solvent because of its low purchase and disposal costs, it is also highly effective in extracting the silymarin compounds from milk thistle seed. For each of the compound, extraction with 100°C water gave the highest yield and concentration. After 210 min of extraction at 100°C, the yield of taxifolin was 1.2 mg/ g of seed, while the yields of silychristin, silybinin A, and silybinin B were 5.0, 1.8, and 3.3 mg/g of seed, respectively. The ratios of the extracted compounds, and particularly the ratios at long extraction times, showed that the more polar com-

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