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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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EXTRACTION OF SILYMARIN COMPOUNDS FROM MILK THISTLE (*SILYBUM MARIANUM*) SEED USING HOT, LIQUID WATER AS THE SOLVENT

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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 often occur. When compared to traditional solvents, water can be viewed as 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 hepatoxic 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 per g of seed, while the yields of silychristin, silybinin A and silybinin B were 5.0, 3.7 and 6.5 mg per g of seed, respectively. The overall diffusion coefficients for the four compounds increased with temperature and ranged from 0.14×10^{-10} to 4×10^{-10} m²/sec, indicating that the diffusion coefficients could potentially be used for quantitative comparisons of extraction conditions. 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.

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 10 feet with dark and shiny leaves, and purple to reddish flowers. Milk thistle has an indeterminate growth habitat, 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 (Figure 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).

The Deutsches Arzneibuch procedure for silymarin extraction is a two-step process in which seeds are first defatted in a Soxhlet extraction with petrol for 4 hr, followed by a second Soxhlet extraction with methanol for 5 hr. Using this procedure, reported silybinin yields were 11 mg of silybinin per g of seed (Benthin *et al.*, 1999). Milk thistle was also extracted using pressurized liquid extraction techniques, in which 12 mg of silybinin per 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 22.2 mg per 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 content 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 problems. The lack of consistency among products can be due in part to the extraction step, in which 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 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; Kub·tov· *et al.,* 2001). Water is 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 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 and 498 K (hot/liquid) water, where increases were observed with temperature. Kub·tov· *et al.* (2001) showed that the extraction of peppermint compounds using (hot/liquid) water at 175°C required 15 min, as compared to 4 hr with hydrodistillation. The use of (hot/liquid) water as an extraction solvent shows promise as the search for milder and environmentally friendly solvents is intensified.

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, and consists of a first step toward process characterization. Silymarin compounds, ranging from highly polar (taxifolin) to less polar (silybinin), were extracted in 50-100°C water over 17 hr. From their concentrations, overall diffusion coefficients (D_s) were calculated and used as a parameter for evaluating the effectiveness of the extraction conditions.

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 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, VA) set at 80 strokes per 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.

Samples of extraction water were taken in triplicate every 30 min, including time zero, using a 1 mL pipette. Time zero 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, 85°C). To estimate the silymarin concentrations at time infinity, a final sample was taken after 300 min for the 100°C experiments and 1020 min for the 50°C, 70°C and 85°C experiments. The aliquots were placed in preweighed test tubes and weighed to determine aliquot weight. Subsequently, the aliquots were evaporated to dryness in a SpeedVac (Savant Instruments, Holbrook, NY). 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, MA) composed of an Alliance 2690 separations module and a 996 Photodiode Array, controlled with Millennium[32] chromatography software. Separation of the silymarin compounds was obtained using a SymmetryÆ (Waters, Milford, MA) C_{18} pre-column placed in series with a SymmetryÆ (Waters, Milford, MA) C_{18} 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, 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 (the first peak) and silybinin B (second peak). A sample chromatogram from the extraction of milk thistle seeds is shown in Figure 2. The HPLC procedure was previously described by Wallace *et al.,* (2002a).

Mathematical Model

The overall diffusivities of the compounds (D_s) were determined using the method of Schwartzberg (1975), who presented a series solution to Fick's second law of diffusion:

$$\frac{C_\infty - C}{C_\infty} = \sum_{n=1}^{\infty} B_n \exp\left[\frac{-q_n^2 D_s t}{a^2}\right] \qquad (1)$$

In Equation (1), a is the particle size (mm),   is the concentration of the selected silymarin at infinite time (mg/ml),

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C is the concentration of the selected silymarin at any given time (mg/ml), B_n and q_n are parameters related to the geometry of the solid particles, D_s is the solute diffusivity (m²/s) and t is the immersion time (min). Table 1 presents a summary of the constants used in the mathematical model, along with their corresponding values in these experiments.

The solution of Equation (1) may be approximated by the first term in the series for $Dt/a^2 > 0.1$. Thus, a plot of $\boxed{x}$ vs. time yields a straight line with a slope that corresponds to the following expression:

$$-\frac{D_s q_1^2}{2.303a^2} \qquad (2)$$

If the particle is considered a sphere, q_1 can be obtained by iteration through the following equation:

$$\tan q_1 = \frac{3q_1}{3 + \alpha q_1} \qquad (3)$$

where a is the stripping factor related to solid and liquid volume and equilibrium ratio. Thus, D_s is obtained by combining Equations (2) and (3) with the constants of Table 1.

Results and Discussions:

For all temperatures, three distinct experiments were conducted, of which three samples were taken per time point (total of nine samples per time point). Figure 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.

Figure 4 shows typical results from the extraction of taxifolin, silychristin, silybinin A and silybinin B, presented as the yield of each compound (mg per 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 per g of seed, while the yields of silychristin, silybinin A and silybinin B were 5.0, 3.7 and 6.5 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, 3.7 and 6.7 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 performed by Wallace *et al.* (2002a).

Table 2 shows overall diffusion coefficients for the compounds as a function of temperature. The diffusion coefficients increased with temperature, indicating that the diffusion coefficients could potentially be used for quantitative comparisons of extraction conditions, including various water temperatures and different solvents. The calculated coefficients for water extraction fall within the spectrum of diffusion coefficients obtained for the extraction of sucrose in water (Schwartzberg and Chao, 1982).

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 (sampling points) are shown in Figure 5. These temperatures were chosen because the flavanolignan concentrations were not as large at temperatures below 85∞C. As is noted in Figure 5a, at 85°C the ratio of taxifolin to silybinin B increased rapidly to 0.35 g/g and then held constant at that level. At 100°C, the ratio reached a maximum of 0.32 g/g and then gradually fell with time to 0.18 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 Figure 5b. At 85°C, the ratio rapidly rose to just above 0.9 g/g, and then gradually increased before leveling out at 1.1 g/g. At 100°C, the ratio increased to a maximum of 1.0 g/g, and then gradually fell to 0.78 g/g. The data of Figure 5c 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 B) are preferentially extracted at 100°C (see also the data of Table 3). The data reported by Wallace *et al.* (2002a) showed that the taxifolin to silybinin B, the silychristin to silybinin B and the silybinin A to silybinin B ratios were 0.02, 0.1 and 1.2. 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 is necessary in the extraction procedures proposed by Kahol *et al.* (2001) and by Benthin *et al.* (1999). The work of Wallace *et al.* (2002b) will compare the extraction of non-defatted and defatted milk thistle seed meal using ethanol, as the solvent, will hopefully shed more light on this subject mater.

Conclusions:

Water is not only an interesting alternative solvent because of its low operating and disposal costs, but is also highly effective in extracting the silymarin compounds from milk thistle seed. For each of the compounds, 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 per g of seed, while the yields of silychristin, silybinin A and silybinin B were 5.0, 3.7 and 6.5 mg per g of seed, respectively. The overall diffusion coefficients for the four compounds increased with temperature and ranged from 0.1-4.0 x 10^{-10} m^2/sec, indicating that the diffusion coefficients could potentially be used for quantitative comparisons of extraction conditions. 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 compounds (silybinin A and B) were preferentially extracted at 100°C.

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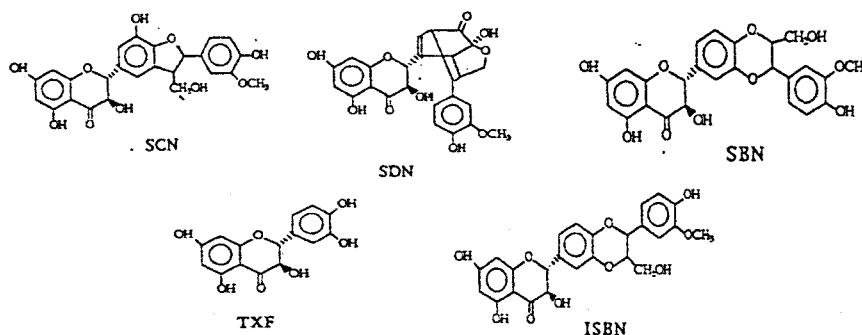
Figure 1: *Structures of silychristin (SCN), silydianin (SDN), silybinin (SBN), taxifolin (TXF) and isosilybinin (ISBN).*
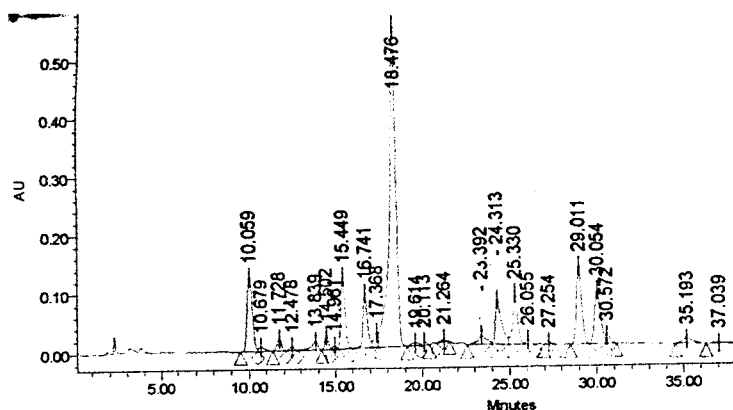
Figure 2: Typical chromatogram of milk thistle seed extract. Retentions times of taxifolin, silychristin, silydianin, silybinin A and silybinin B were 10.059, 18.476, 21.264, 24.313 and 25.330 minutes, respectively. It should be noted that this particular seed lot contained minuscule amounts of silydianin.

Constant	Notation/Equation	Value
Reciprocal of moisture content	m	40
Liquid volume (cm^3)	L	200
Density of powder (g/cm^3)	F	0.4
Solid volume (cm^3)	α=Lm/F	5.1
Stripping factor		1540
Parameter from Eq.3		0.93
Concentration at infinite time (mg/mL)	C_∞	
Taxifolin		1.304
Silychristin		5.541
Silybinin A		3.805
Silybinin B		7.362

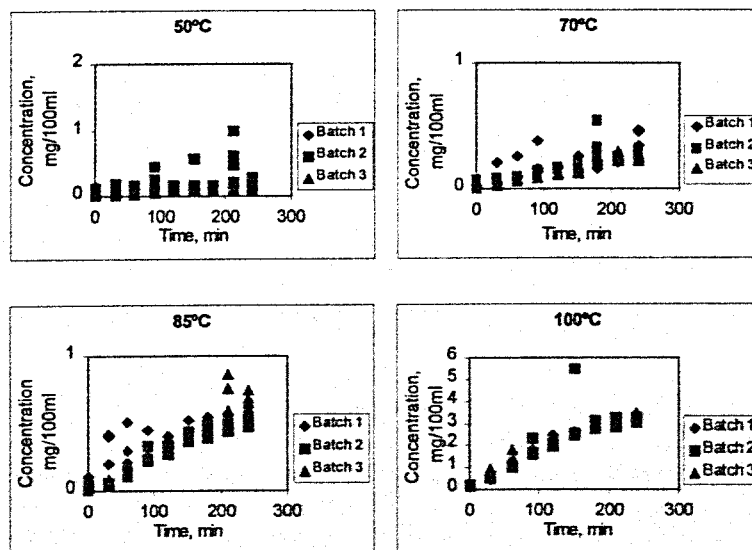Table 1: Values used to calculate diffusion coefficients

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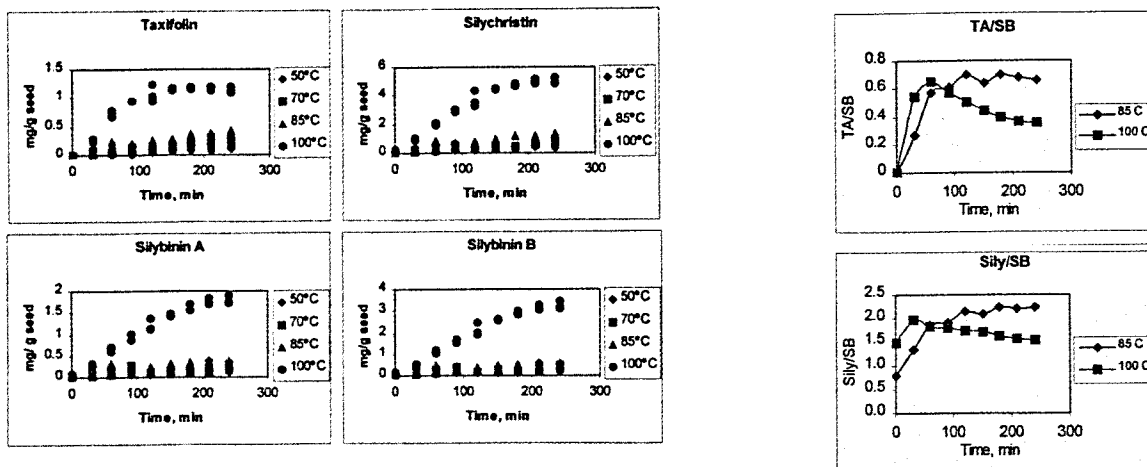
Figure 4: Concentration in mg of compound per gram of seed as a function of time at different temperatures. Results based on the first batch of each temperature. The maximum time reported is 240 min; experimentally, there were infinite times of 300 min for 100°C, and 1205 min for 50°C, 70°C and 85°C.
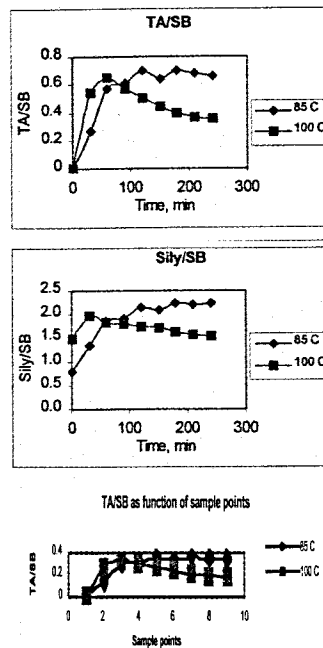

Figure 5 Compound ratio as a function of sampling points for the 85°C and 100°C experiments. Top (A) taxifolin to silybinin B ratio. Middle(B) silychristin to silybinin B ratio. Bottom (C) Silybinin A to silybinin B ratio.

Water Temperature (°C)	Diffusion Coefficient (m^2/s*10^{-10})			
	Taxifolin	Silychristin	Silybinin A	Silybinin B
50	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.8 ± 0.0
70	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
85	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
100	3.0 ± 1.0	4.0 ± 1.0	3.8 ± 0.8	2.7 ± 0.8

Table2: Diffusion coefficients (D$_s$) calculated for silymarin present in milk thistle seed

Water Temperature (°C)	Dielectric Constant ε	Ratio of Compound to Silybinin B		
		Taxifolin/SB	Silychristin/SB	Silybinin A/SB
50	70	0.281	0.910	0.615
70	64	0.297	0.935	0.639
85	54	0.330	1.118	0.630
100	56	0.176	0.772	0.551

Table 3: Calculated ratio compound/Silybinin B as a function of temperature. These ratios were calculated at the last sampling point.

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Faculty Comments:

Dr. Danielle Julie Daniels, who worked in the laboratory with Mr. Alvarez-Barreto, made the following remarks about his work:

I have not taught Mr. Barreto, hence I do not know him from a classroom perspective. However, I have worked closely with Mr. Barreto in a laboratory setting. Honesty, intelligence, rigor and hard work characterize his laboratory performance. Although this student is beginning his research career, he displays scientific maturity and understanding well beyond what could be expected from his peer reference group. As Mr. Barreto grasped the mechanics of the experiments, he became independent in terms of laboratory autonomy, generating reproducible and high quality data. This proved to be an invaluable asset for Dr. Clausen and myself. These same data sets served as a platform on which a NRI/USDA proposal was prepared and submitted.

Once the data were generated, Mr. Barreto actively participated in its analysis and spent a large part of his Christmas holiday generating a first draft of this paper. As is the due course of scientific writing, this paper bounced back and forth between Mr. Barreto and his mentors. Mr. Barreto will undoubtedly work in a research setting, as he will continue his work as a chemical engineering graduate student at the University of Oklahoma. My only regret is that we will lose a great asset.

Mr. Alvarez-Barreto's other mentor, Dr. Edgar Clausen, had this to say about his efforts:

Mr. Barreto transferred from the Universidad de Carabobo in Venezuela in the Spring, 2001 semester, largely because his mother entered the Ph.D. program at the U. of A. in food science. I became acquainted with Jose during this semester as his instructor in two undergraduate chemical engineering classes, and he subsequently decided to do an undergraduate research project for academic credit under my direction in the summer of 2001. Like many research projects, we had just "skimmed the surface" of the project by the end of the summer, but funds were not available to continue the work. Mr. Barreto volunteered to continue this now better-defined project on hot, liquid water extraction of flavanolignans *from* milk thistle throughout the 2001-2002 academic year without pay under the direction of Dr. Julie Carrier and me. This work built upon previous analytical work with milk thistle by Dr. Carrier, and the pioneering work of Hawthorne *et al.* in using hot, liquid water as an alternative solvent for the solubilization of both polar and non-polar compounds.

Mr. Barreto has done a good job on this project, including setting up the experiments and obtaining

the experimental data, analyzing the data and preparing the manuscript. The analytical support was provided by Dr. Carrier. Dr. Carrier and I both believe that this is important work that may open the door for the use of water as an important extraction solvent. As a result of these efforts, Mr. Barreto earned a $500 undergraduate research award from the College of Agriculture, and his work will be subsequently published in *Discovery*, a publication of undergraduate research in agriculture. In addition, Mr. Barreto will present his work in April at the Mid-America Regional AIChE Student Paper Contest at the University of Iowa. His research effort was the foundation for a USDA NRI research proposal and will be a significant part of an additional refereed research publication. The research efforts are currently being expanded to examine high temperatures for hot, liquid water extraction, as well as the application of hot water extraction to other natural products. I was so impressed by Mr. Barreto's dedication in the classroom and laboratory that I arranged for him to get a small scholarship from the Department of Chemical Engineering to help ease the financial burden of out-of-state tuition. Dr. Carrier and I were recently awarded a small research contract on neutraceuticals extraction from natural products, and Mr. Barreto was the natural choice for doing the laboratory work, this time with compensation. Mr. Barreto will be attending graduate school in chemical engineering in the fall at the University of Oklahoma. We will be losing a valuable research colleague who has a bright future.