Appl Biochem Biotechnol (2009) 158:362–373
DOI 10.1007/s12010-009-8594-z

Extraction of Co-Products from Biomass: Example of Thermal Degradation of Silymarin Compounds in Subcritical Water

Lijun Duan · Sunny N. Wallace · Abigail Engelberth · Justin K. Lovelady · Edgar C. Clausen · Jerry W. King · Danielle Julie Carrier

Received: 22 May 2008 / Accepted: 24 February 2009 /
Published online: 10 March 2009

Abstract In an effort to increase revenues from a given feedstock, valuable co-products could be extracted prior to biochemical or thermochemical conversion with subcritical water. Although subcritical water shows significant promise in replacing organic solvents as an extraction solvent, compound degradation has been observed at elevated extraction temperatures. First order thermal degradation kinetics from a model system, silymarin extracted from *Silybum marianum*, in water at pH 5.1 and 100, 120, 140, and 160 °C were investigated. Water pressure was maintained slightly above its vapor pressure. Silymarin is a mixture of taxifolin, silichristin, silidianin, silibinin, and isosilibinin. The degradation rate constants ranged from 0.0104 min^{-1} at 100 °C for silichristin to a maximum of 0.0840 min^{-1} at 160 °C for silybin B. Half-lives, calculated from the rate constants, ranged from a low of 6.2 min at 160 °C to a high of 58.3 min at 100 °C, both for silichristin. The respective activation energies for the compounds ranged from 37.2 kJ/gmole for silidianin to 45.2 kJ/gmole for silichristin. In extracting the silymarin with pure ethanol at 140 °C, no degradation was observed. However, when extracting with ethanol/water mixtures at and 140 °C, degradation increased exponentially as the concentration of water increased.

Keywords Extraction · Kinetics · Silymarin · Subcritical water · Thermal degradation

L. Duan · S. N. Wallace · A. Engelberth · J. K. Lovelady · E. C. Clausen · J. W. King
Ralph E. Martin Department of Chemical Engineering, University of Arkansas,
3202 Bell Engineering Center, Fayetteville, AR 72701, USA

D. J. Carrier (✉)
Department of Biological and Agricultural Engineering, University of Arkansas,
203 Engineering Hall, Fayetteville 72701, USA
e-mail: carrier@uark.edu

Introduction

The economic competitiveness of cellulosic ethanol production is highly dependent on feedstock cost, which ranges from 35–50% of the total ethanol production costs [1]. In addition to a $30–$36 per dry ton payment to the producer, harvesting, storage, and transportation costs are estimated at $40–$48 per dry ton of biomass, depending if the feedstock is harvested as a bale, a loaf or ensiled [2].

In an effort to increase revenues from a given feedstock, co-products could be extracted prior to the biochemical or thermochemical conversion at the site of the biorefinery or at a site of close proximity. McAloon et al. [3] outlined the unit operations for co-product production in the biorefinery in terms of energy. Beer column bottoms, consisting largely of lignin, will be obtained from the processing of fermentation solids and will be dewatered in a triple-effect evaporator before being recovered and combusted in a fluidized bed combustor. Lynd et al. [4] showed that the thermochemical conversion of fermentation waste products to heat or electricity enhances the economics of the cellulosic biorefinery. Aside from energy production, McAloon et al. [3] reported that the transformation of lignin into higher-value co-products is important to the long-term commercial viability of the biorefinery.

In addition to thermochemical conversions, co-products in the form of valuable phytochemicals could be extracted with subcritical water prior to the biochemical or thermochemical conversion on- or off-site of the biorefinery [5]. Either from a biochemical or a thermochemical biorefinery, these phytochemicals could find use in human and animal health care products, cosmetic applications, and as essential ingredients in green cleaning products, as there is a growing preference among consumers for phytochemicals in the foods they consume and in the personal care and household products they utilize. However, for extraction of co-products to be possible, the extraction must be carried out with a solvent that is compatible with biorefinery operations. Subcritical water can be such a solvent.

Subcritical water extraction of phytochemical co-products circumvents the use of organic solvent extraction [5], which has been the traditional method of removing co-products from plant material. Water is less costly to purchase, does not have to be recovered, is less costly to dispose of, and residual traces of water in the extract are not toxic. However, in developing innovative extraction processes, limits to technology can be encountered, since the use of subcritical water extraction may generate byproducts. Thermal treatment can sometimes not only decrease the yield of the desired extractant, but can also yield degradation products which may render the extracted compounds ineffective or even harmful. An understanding of the quantitative effects of temperature on subcritical water extraction is essential in selecting the optimal conditions for extraction.

This paper defines first order degradation kinetics of phytochemicals, namely the model compound silymarin contained in milk thistle, as a function of subcritical water extraction temperature. Additionally, this work examines the effect of solvent composition (*i.e.* water, ethanol, and water–ethanol) on the formation of degradation products.

Materials and Methods

Plant Material Milk thistle seeds were purchased from Frontier Herbs (Norway, IA, USA). The seeds were stored at −20 °C before being ground to a particle size of 0.4 mm as detailed in ASAE S319.1 [6].

Subcritical Water Extraction A hot/liquid water dynamic extraction device was constructed from the work of Miller and Hawthorne [7] and used for subcritical extraction of milk thistle seed meal; a schematic of the oven is displayed in Fig. 1. Approximately 0.5 g of the seed meal were packed into the biomass cartridge (6.4 mm i.d., 9.5 mm o.d., 67.1 mm length). Glass wool was placed at the biomass cartridge outlet to prevent plugging of the cell frit. After the loaded biomass cartridge was installed in a GC oven, water was pumped through the cell at a constant flow rate via the preheating coil. When water was observed at the collection exit, methanol flow was initiated to keep the cooled silymarins in solution. A pressure regulator was adjusted to keep the pressure above the saturation pressure of water at the desired temperature selected in the GC oven. When the desired temperature was reached, continuous sample collection began using preset time intervals for each sample tube. After collection, 1 ml of each sample was evaporated to dryness with nitrogen in a self-constructed evaporator. The solids were re-dissolved in 1 ml of methanol, filtered (0.45 μm), and analyzed by HPLC as discussed below [8].

In order to establish the formation of degradation products, 5.5 mg of silibinin was extracted with 200 ml of de-ionized water in a stainless steel vessel (# 452HC3) from Parr Instrument Company (Moline, IL, USA). Extraction parameters included: T=120 °C, P=411 kPa (pressurized using nitrogen), and an agitation rate of 150 rpm [9]. The silibinin was added at time zero and the extraction time of 30 min was started when the water temperature reached 120 °C [8, 9].

Silymarin Degradation Water for the silymarin degradation studies was obtained from a Milli-Q TM Water System (Millipore, Bedford, MA, USA), and degassed by boiling for 15 min, while methanol was secured from Honeywell International Inc. (Muskegon, MI, USA). Silymarin compound degradation studies were carried out batchwise at 100, 120, 140, and 160 °C in 36 stainless steel tubes (2.0 mm i.d., 3.2 mm o.d., 152 mm length, 0.5 cm^3 volume). The tubes were completely filled with the silymarin/water mixture and sealed with end caps on both sides to keep the water in its liquid state. The pH of the liquid was measured with a pH meter from Orion (Beverly, MA, USA) before and after the experiments. At the beginning of a typical degradation experiment, the 36 tubes were placed in an oven at the desired temperature. Six tubes were removed simultaneously from the oven every 10 min for 1 h, and quickly quenched in ice water. The contents of the six tubes were combined, evaporated, and reconstituted in methanol for HPLC analysis [8, 10].

Standards Silymarin, which contains silybin A and B, silidianin, silichristin, and isosilibinin A and B, was obtained from Sigma (St. Louis, MO, USA). Silymarin contains taxifolin (TAX); and this standard was obtained from PhytoLab (Hamburg, Germany). Silibinin was obtained as a mixture of silybin A (SA) and silybin B (SB) from Sigma (St. Louis, MO, USA). Silichristin (SC) and silidianin (SD) standards were obtained from

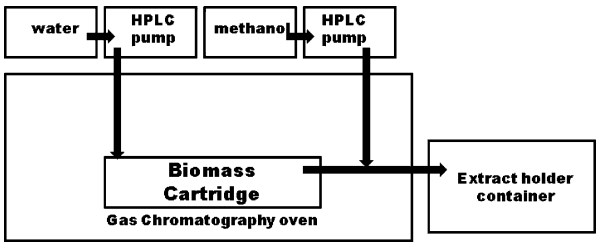

Fig. 1 Schematic of subcritical water extraction set-up for dynamic extraction

PhytoLab (Hamburg, Germany). At the time that this study was conducted, no standard was available for isosilibinin.

HPLC Analysis Silymarins were analyzed by HPLC as described by Wallace et al. [8, 10] using a Waters Instrument, equipped with a 2996 photodiode array detector and a 2695 separations module, and controlled with Empower software. Separation of the silymarin compounds was accomplished using a Symmetry® (Waters, Milford, MA, USA) C_{18} pre-column placed in series with a Symmetry® (Waters, Milford, MA, USA) C_{18} column (150× 4.6 mm, 5 μm), both at 40 °C. A 10-μL sample volume was injected. Solvent A consisted of 20:80 methanol–water, while solvent B consisted of 80:20 methanol–water. The gradient 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. Solvent A–solvent B was then held constant at proportions of 45:55 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.

Data Analysis The first order degradation rate constants (k) were determined from plots of the experimental data using the natural log of the ratio of the compound concentration to its initial concentration as a function of time, while activation energies (E) were calculated from the slope of a plot of the natural log of k as a function of the inverse absolute temperature. Compound half-lives were calculated as the time required for the silymarin concentrations to decrease to half of their initial values, in accordance with the first order rate constants. Finally, the effect of ethanol concentration on the degradation of silymarin in aqueous ethanol solutions was modeled as an exponential relationship, determined from a plot of the natural log of k as a function of the ethanol concentration (vol %).

Results and Discussion

Dynamic Extraction Although silymarin does not readily dissolve in room temperature water, it can be extracted from ground milk thistle seeds with subcritical water [9]. Silymarin ratios and concentrations have been observed to vary from seed batch to seed batch [10]. The batch of seeds that was extracted in this work contained, in decreasing concentration order, SC, SB, SA, and TAX. No SD was detected in this seed batch. The pH of the mixture at the beginning of each water degradation experiment was 5.1 and did not vary during the course of the study. Figure 2 shows the cumulative yields of the silymarin compounds in milligram per gram of dry seed as a function of time at temperatures of 100, 120, and 140 °C. Although not shown, as the extraction temperature was increased from 100 to 120, and finally to 140 °C, the time required to extract the majority of the compounds (referred to as the peak extraction time) decreased. Even though extraction time could be decreased at increased temperature, higher overall silymarin yields, particularly higher yields of SC and TAX, were obtained at 120 °C. The higher yields that were obtained at 120 °C are indicative that compound loss was occurring during the dynamic extraction process.

Decreasing concentrations or yields of extractants were also observed with increasing temperature in subcritical water extraction systems when extracting paclitaxel from the bark of the Pacific yew (*Taxus cuspidata*) [11]; essential oils from marjoram (*Thymus mastichina*) leaves [12]; iridoid glycosides from the leaves of *Veronica longifolia* [13]; 1,1-diphenyl-2-picrylhydrazyl free radical scavenging compounds from Taiwan yams

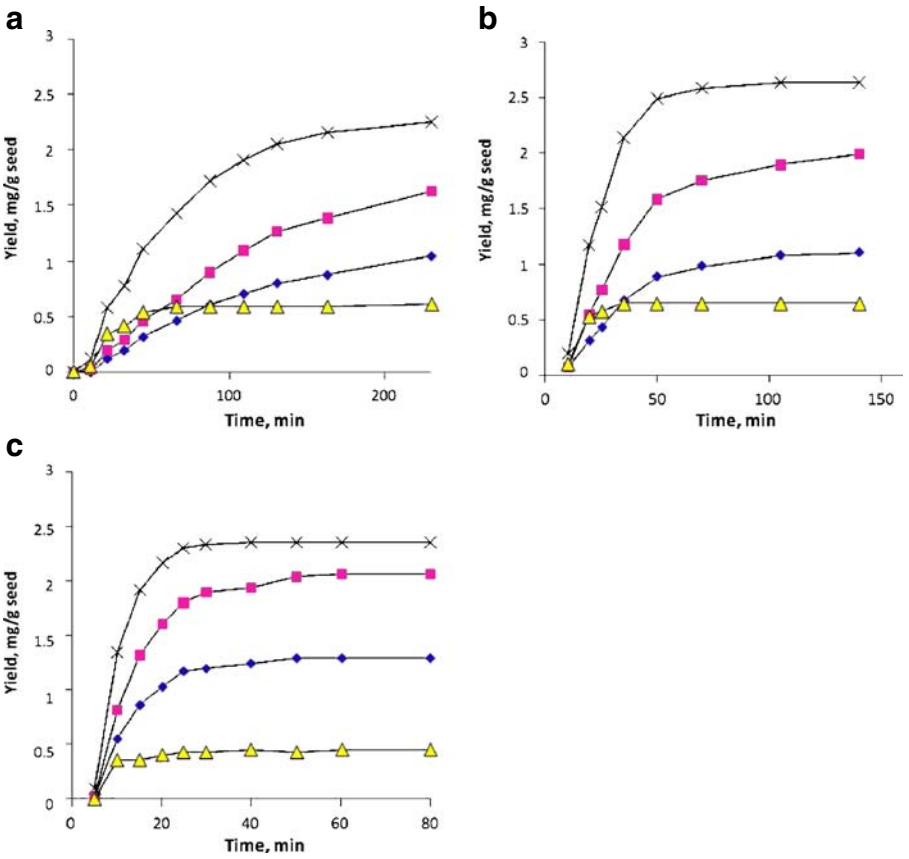

Fig. 2 Cumulative extraction yields of silymarin compounds and taxifolin as a function of time and temperature at **a** 100 °C, **b** 120 °C, and **c** 140 °C in subcritical water (silybin A (*filled diamond*, SA), silybin B (*filled square*, SB), silichristin (*multiplication symbol*, SC), taxifolin (*unfilled triangle*, TAX))

(*Dioscorea alata*) [14]; and antioxidant compounds from rosemary plants (*Rosmarinus officinalis* L.) [15]. As an example, the yield of the iridoid glycoside, catapol, at 100 °C and 150 kg/cm^2 was 2.6% (*w*/*w*) dry biomass, while the yield at 150 °C and 150 kg/cm^2 was 0.9% (*w*/*w*) dry biomass [13]. The decreased yields, as the temperature was further increased, may be due to compound degradation. Although degradation kinetics are not available for subcritical water phytochemical extraction systems, degradation kinetic parameters have been developed for related systems, such as chlorophyll degradation in broccoli juices during high temperature/high pressure processing [16].

Degradation of Silymarin Isolates Figure 3 shows the HPLC-UV trace of silibinin both before (Fig. 3a) and after (Fig. 3b) extraction for 30 min with 120 °C subcritical water. SA and SB eluted at retention times of 24 and 25 min (Fig. 3a), respectively; and the heat-treated silibinin showed distinct peaks at retention times of 3, 19, 21, and 26 min, in addition to the SA and SB peaks. Although not presented in this work, the other silymarins also yield multiple distinct degradation products that are currently being characterized by LC/MS-MS analyses. Therefore, individual solutions of silymarin components were

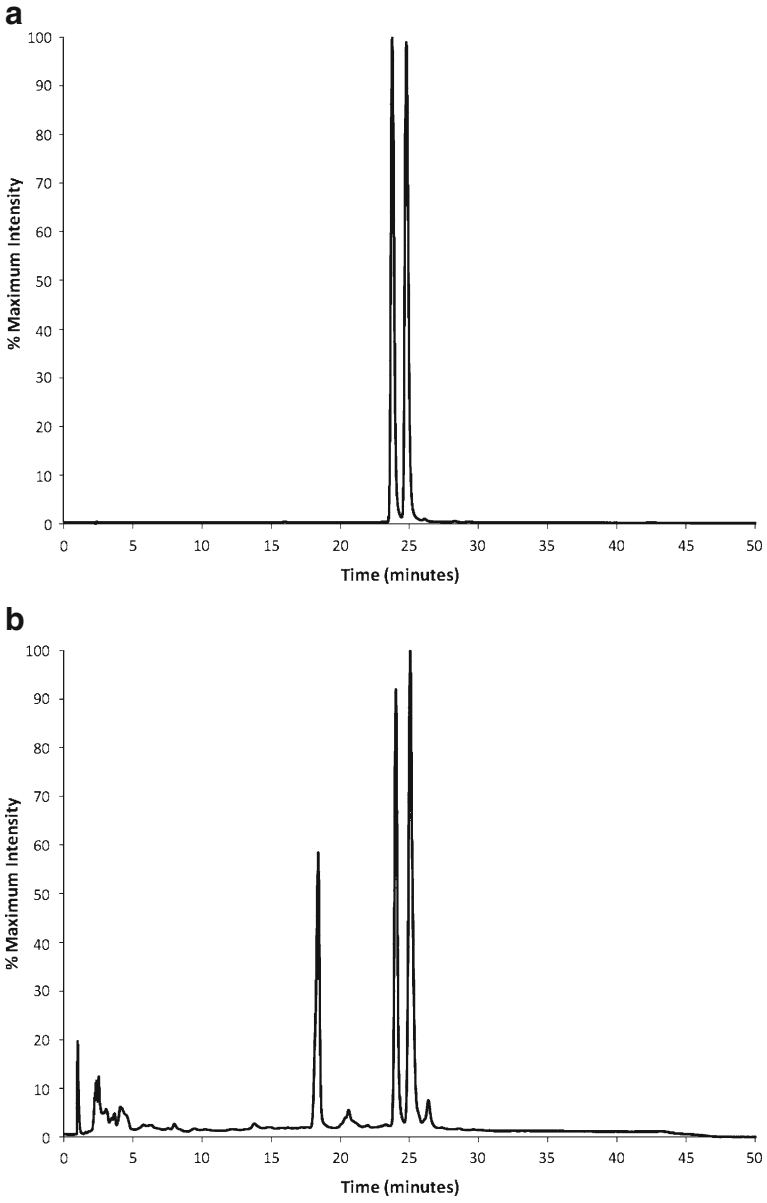

Fig. 3 HPLC-UV traces of silibinin before extraction with 120 °C subcritical water (**a**) and after extraction with 120 °C subcritical water (**b**)

prepared with reference compounds and their stability was tested in batch reactors (stainless steel tubes) for up to 60 min at temperatures up to 160 °C. Figure 4 presents concentration plots for each of the silymarin compounds as a function of the subcritical water temperature. The pH of the mixture at the beginning of each water degradation experiment was 5.1, and did not vary during the course of the study. In examining these plots,

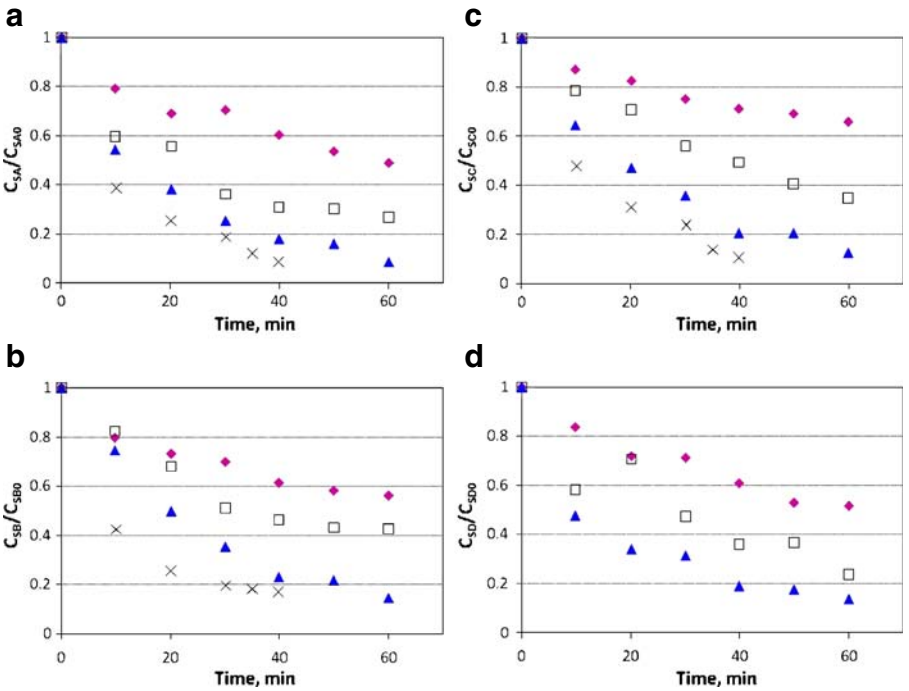

Fig. 4 Degradation of silymarins in subcritical water at pH 5.10 at 100 °C (*filled diamond*), 120 °C (*unfilled square*), 140 °C (*filled triangle*), and 160 °C (*multiplication symbol*). **a** silybin A (SA); **b** silybin B (SB); **c** silichristin (SC); **d** silidianin (SD)

compound degradation expressed as decreasing $\frac{C_A}{C_{A0}}$ occurred with time and was more pronounced at increased temperature. As an example, less than 20% of SC was degraded in 20 min at 100 °C, while about 35% degraded in 60 min. At 160 °C, almost 70% of SC degraded in 20 min, while nearly 90% degraded in 40 min. Similar results were obtained for SA, SB, and SD.

Degradation kinetics are most often modeled as simple first order reactions:

$$-r_A = -\frac{dC_A}{dt} = k\, C_A \tag{1}$$

where $-r_A$ is the rate of disappearance of the silymarin compound, with time, t, in g/l·min; C_A is the concentration of the silymarin in g/l; and k is the degradation rate constant in min^{-1}. Separating the variables of Eq. 1, integrating, and plotting $\ln\frac{C_A}{C_{A0}}$ vs. t enables the determination of k as the negative slope of the plot as shown in Fig. 5.

The calculated degradation constants, k, for the silymarins extracted at 100, 120, 140, and 160 °C are presented in Table 1. As noted, the rate constants ranged from a low of 0.0104 min^{-1} for SC at 100 °C to a maximum of 0.0840 min^{-1} for SB at 160 °C. The degradation rate constants of the silymarin compounds for the most part doubled when the temperature was increased from 100 to 120 °C; and was increased by a factor of 8 when the temperature was increased from 100 to 160 °C. Compound half lives may be calculated by setting C_A as one half of C_{A0} and solving for t for a given rate constant. Compound half

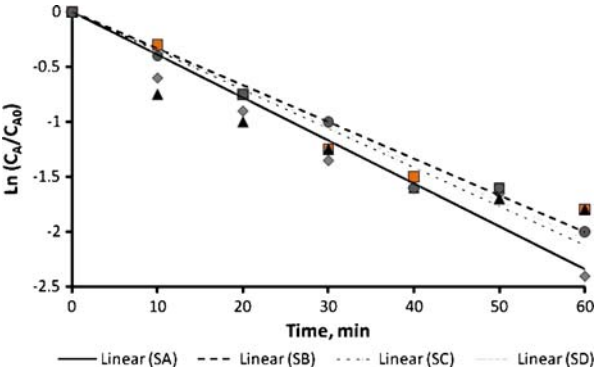

Fig. 5 Determination of degradation rate constants of silymarin compounds in subcritical water at pH 5.10 and 140 °C (silybin A (SA, *filled diamond*), silybin B (SB, *filled square*), silichristin (SC, *filled triangle*), silidianin (SD, *filled circle*))

lives are summarized in Table 2. The half lives ranged from 6.2 min for SC at 160 °C to 58.3 min for SC at 100 °C.

The effects of temperature on the degradation rate constant can be described by the Arrhenius law. Rearrangement of the Arrhenius law yields:

$$\ln k = \ln k_0 - E/RT \tag{2}$$

where k_0 is the frequency factor in min^{-1}, E is the activation energy in kJ/gmole, R is the ideal gas constant (8.314×10^{-3} kJ/gmole·K), and T is the absolute temperature in K.

Thus, a plot of ln k vs. $1/T$ yields a straight line of slope $-E/R$, from which the activation energy may be calculated. The activation energies for the compounds are summarized in Table 3. As noted, they were quite close to each other, ranging from 37.2 kJ/gmole for SD to 45.2 kJ/gmole for SC. The activation energies for the degradation of silymarin compounds in subcritical water were of the same order of magnitude as the activation energies for the degradation of chlorophyll a and chlorophyll b and monoammonium glycyrrhizinate in water at atmospheric pressure [16, 17].

Silymarin Degradation in Ethanol Solutions Under Similar Conditions Silymarin compound degradation studies were also carried out in 100% ethanol at 140 °C using the stainless steel tubes; results are shown in Fig. 6. Unlike the degradation that was observed

Table 1 First order degradation rate constants *(k)* and corresponding standard deviations of silymarin compounds at pH 5.10 and 100, 120, 140, and 160 °C.

	Degradation rate constants, k (min^{-1}) (10^{-2})			
Compound	100 °C	120 °C	140 °C	160 °C
Silybin A	1.11±0.21	2.39±0.25	4.05±0.08	8.06±4.02
Silybin B	1.13±0.03	1.88±0.23	3.83±0.80	8.40±5.81
Silichristin	1.04±0.37	1.97±0.23	4.05±0.86	8.34±4.04
Silidianin	1.42±0.31	2.83±0.66	4.78±1.65	–[a]

[a] Silidianin was not well identified and quantified

Table 2 Silymarin half lives at pH 5.10 and 100, 120, 140, and 160 °C.

	Compound half life (min)			
Compound	100 °C	120 °C	140 °C	160 °C
Silybin A	50.7±16.5	25.0±5.7	12.6±6.4	7.8±3.0
Silybin B	51.2±14.4	32.0±7.0	14.1±5.7	6.4±2.0
Silichristin	58.3±11.7	29.6±7.9	13.6±5.0	6.2±3.0
Silidianin	39.4±13.3	18.3±8.8	11.8±3.9	–[a]

[a] Silidianin was not well identified and quantified

Table 3 Activation energies of silymarin compounds at pH 5.10.

Compound	Activation energy, *E* (kJ/mol)
Silybin A	40.0±7.5
Silybin B	38.3±9.4
Silichristin	45.2±2.0
Silidianin	37.2±2.5

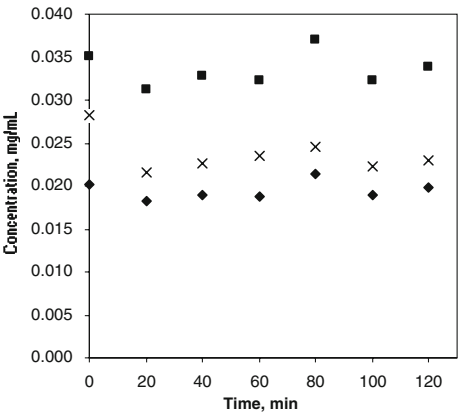

Fig. 6 Concentrations of silymarin compounds in ethanol (100% *v*/*v*) during treatment at 140 °C (silybin A (SA, *filled diamond*), silybin B (SB, *filled square*), silichristin (SC, *multiplication symbol*))

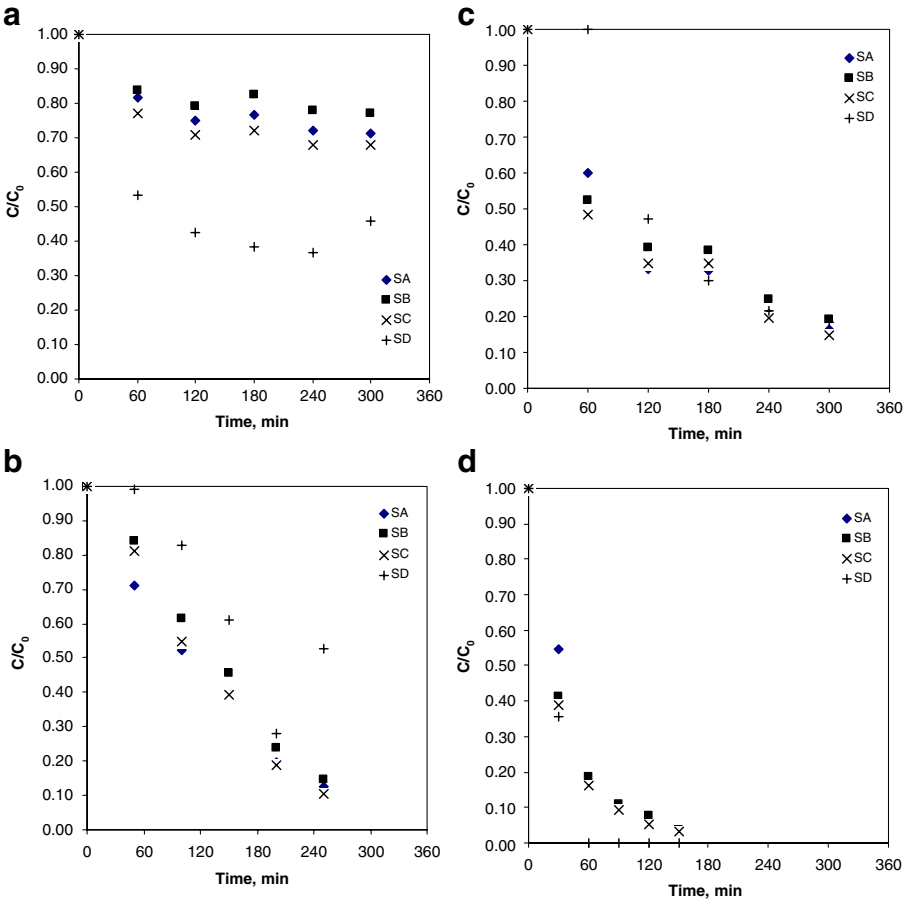

Fig. 7 Degradation of silymarins (silybin A (*filled diamond*, SA), silybin B (*filled square*, SB), silichristin (*multiplication symbol*, SC), silidianin (*plus symbol*, SD)) at pH 5.10 and 140 °C in **a** 95% *v*/*v* ethanol; **b** 50% *v*/*v* ethanol; **c** 25% *v*/*v* ethanol; **d** 5% *v*/*v* ethanol

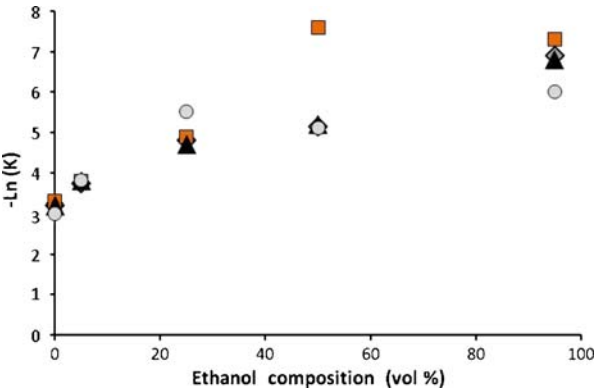

Fig. 8 Determination of degradation rate constants of silymarin compounds in aqueous ethanol solutions at pH 5.10 and 140 °C (silybin A (SA, *filled diamond*), silybin B (SB, *filled square*), silichristin (SC, *filled triangle*), silidianin (SD, *filled circle*)

in pure subcritical water (Fig. 4), the concentrations of the silymarin compounds remained essentially constant with time (little to no degradation) in the presence of 100% ethanol. Figure 7 shows the effect of intermediate ethanol concentrations (5%, 25%, 50%, and 95%, *v*/*v*) on silymarin compound degradation at 140 °C. Generally, as the percentage water in the extracting solvent increased, the degradation of the compounds increased as well. For example, SC was 30% degraded in 120 min in 95% ethanol and 95% degraded in 120 min in 5% ethanol. However, degradation in 50% ethanol was actually greater than in 25% ethanol. For example, SB was about 75% degraded in 180 min in 50% ethanol and only 60% degraded in 120 min in 25% ethanol. Further study will be required to find an ethanol/water ratio where the degradation rate of the flavonolignans is minimized. The work of Bilia et al. [18] may shed light on this discussion. They found that silymarin compound degradation was less in 60% (*v*/*v*) milk thistle tinctures in comparison to 40% (*v*/*v*) milk thistle tinctures at 25 °C.

In general, the silymarin compounds degraded similarly at a given concentration of ethanol in the solvent. This effect is quantified in Fig. 8, where the natural log of the rate constant for the degradation of each of the compounds is plotted as a function of the ethanol concentration (*v*/*v*). This plot shows that the degradation of SA in aqueous ethanol can be described by the equation:

$$k_{SA} = 0.031e^{-0.036C_E}, \text{ with } r^2 = 0.97 \quad (3)$$

Similarly, the degradation of SB can be described by the equation:

$$k_{SB} = 0.023e^{-0.045C_E}, \text{ with } r^2 = 0.78 \quad (4)$$

the degradation of SC can be described by the equation:

$$k_{SC} = 0.030e^{-0.035C_E}, \text{ with } r^2 = 0.97 \quad (5)$$

and the degradation of SD can be described by the equation:

$$k_{SD} = 0.023e^{-0.026C_E}, \text{ with } r^2 = 0.66 \quad (6)$$

In these equations, C_E represents the aqueous ethanol concentration (*v*/*v*) and $0 \leq C_E$, vol %≤ 95.

Subcritical water extraction offers many advantages as a "green" solvent over traditional solvent extraction, but compound degradation may occur as a result of the increased temperatures. In commercially applying subcritical water as an extraction solvent, the extraction conditions must be adjusted such that product losses are minimized.

References

1. Hess, R., Wright, C., & Kenney, K. (2007). *Biofuels, Bioproducts & Biorefining*, *26*, 181–190.
2. Kumar, A., & Sokhansanj, S. (2007). *Bioresource Technology*, *98*, 1033–1044. doi:10.1016/j.biortech.2006.04.027.
3. McAloon, A., Taylor, F., Yee, W., Ibsen, K., & Wooley, R. (2000). National Renewable Energy Laboratory. 2000. NREL/TP-580-28893.

4. Lynd, L., Laser, M., Bransby, D., Dale, B., Davison, B., Hamilton, R., et al. (2008). *Nature Biotechnology*, *26*, 169–172. doi:10.1038/nbt0208-169.
5. King, J. (2006). *In Modern extraction techniques: Food and agricultural samples* (pp. 79–95). Washington, D.C.: American Chemical Society.
6. American Society of Agricultural Engineering (ASABE) (2002). *ASAE S319.1. "Method determining and expressing fineness of feed materials by sieving* (49th ed.). USA: St. Joseph.
7. Miller, D., & Hawthorne, S. (2000). *Journal of Chemical & Engineering Data*, *45*, 315–318. doi:10.1021/je990278a.
8. Wallace, S., Raible, J., Carrier, D. J., Vaughn, K., Griffis, C., Clausen, E., et al. (2007). *Canadian Journal of Physiology and Pharmacology*, *85*, 894–902. doi:10.1139/Y07-058.
9. Duan, L., Carrier, D. J., & Clausen, E. (2004). *Applied Biochemistry and Biotechnology*, *114*, 559–568. doi:10.1385/ABAB:114:1-3:559.
10. Wallace, S., Carrier, D. J., Beitle, R., Clausen, E., & Griffis, C. (2003). *Journal of Nutraceuticals, Functional & Medical Foods*, *4*, 37–48. doi:10.1300/J133v04n02_05.
11. Kawamura, F., Kikuchi, Y., Ohira, T., & Yatagai, M. (1999). *Journal of Natural Products*, *62*, 244–247. doi:10.1021/np980310j.
12. Jiménez-Carmona, M., Ubera, J., & Luque de Castro, M. (1999). *Journal of Chromatography. A*, *855*, 625–632. doi:10.1016/S0021-9673(99)00703-7.
13. Suomi, J., Sirén, H., Hartonen, K., & Riekkola, M. (2000). *Journal of Chromatography A*, *868*, 73–83.
14. Chen, P., Tu, Y., Wu, C., Jong, T., & Chang, C. (2004). *Journal of Agricultural and Food Chemistry*, *52*, 1945–1949.
15. Ibañez, E., Kubátová, A., Señoráns, F., Cavero, S., Reglero, G., & Hawthorne, S. (2003). *Journal of Agricultural and Food Chemistry*, *51*, 375–382. doi:10.1021/jf025878j.
16. Van Loey, A., Ooms, V., Weemaes, C., Van den Broeck, I., Ludikhuyze, L., Indrawati, D., et al. (1998). *Journal of Agricultural and Food Chemistry*, *46*, 5289–5294. doi:10.1021/jf980505x.
17. Coiffard, A., Coiffard, J., Peigne, M., & Roeck-Holtzhauer, M. (1998). *Journal of the Science of Food and Agriculture*, *77*, 566–570. doi:10.1002/(SICI)1097-0010(199808)77:4<566::AID-JSFA84>3.0.CO;2-I.
18. Bilia, A. R., Bergonzi, M. C., Gallori, S., Mazzi, G., & Vincieri, F. F. (2002). *Journal of Pharmaceutical and Biomedical Analysis*, *30*, 613–624. doi:10.1016/S0731-7085(02)00352-7.