Silymarin, a mixture of flavonolignans exhibiting many pharmacological activities, is obtained from the fruits of milk thistle (Silybum marianum L. Gaertner). Due to the high lipid content in thistle fruits, the European Pharmacopoeia recommends a two-step process of its extraction. First, the fruits are defatted for 6 h, using n-hexane; second, silymarin is extracted with methanol for 5 more hours. The presented data show that this extremely long traditional Soxhlet extraction process can be shortened to a few minutes using pressurized liquid extraction (PLE). PLE also allows to eliminate the defatting stage required in the traditional procedure, thus simplifying the silymarin extraction procedure and preventing silymarin loss caused by defatting. The PLE recoveries obtained under the optimized extraction conditions are clearly better than the ones obtained by the Pharmacopoeia-recommended Soxhlet extraction procedure. The PLE yields of silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B in acetone are 3.3, 6.9, 3.3, 5.1, 2.6 and 1.5 mg/g of the non-defatted fruits, respectively. The 5-h Soxhlet extraction with methanol on defatted fruits gives only ∼72% of the silymarin amount obtained in 10 min PLE at 125°C.

Introduction

Silybum marianum L. Gaertner, commonly called as milk thistle, blessed milk thistle, Marian Thistle, Mary Thistle or Saint Mary’s Thistle, is an annual or biannual plant from the Asteraceae family. The plant, originally growing in Southern Europe and Asia, is now found throughout the world (1). This troublesome weed is presently cultivated as a medicinal plant and is one of the most important medicinal crops in Europe.

Milk thistle has been used for medicinal purposes for over 2000 years, most commonly for the treatment of liver disease (cirrhosis and hepatitis), as well as for the protection of the liver from toxic substances (2–5). Recent research interest in this plant has been stimulated by studies showing its exceptionally high antitumor activity. Extracts from the plant are now under intense study in the experimental chemoprevention of cancer, and in the amelioration of chemotherapy side effects (6). Recent reports have demonstrated that extracts from this plant are also characterized by many other pharmacological activities, such as anti-inflammatory and antifibrotic effects (2, 7, 8).

The therapeutic effects of milk thistle are closely connected with the presence of the flavonoid complex called silymarin. The mixture consists of silybin A and B, isosilybin A and B, silychristin and silydianin. The highest amount of the complex is present in the fruits of the plant (9–11). The medicinal properties of milk thistle explain why the importance of the flavonolignans analysis has been recognized by researchers, who, so far, have most frequently used high-performance liquid chromatography (HPLC) for this purpose.

The separation of compounds to be analyzed from the plant matrix is the first step in any analysis of medicinal plant constituents. Due to the high contents of lipids in the thistle fruits (∼25%), the silymarin extraction procedure from the matrix involves a two-step process (12). First, the fruits are defatted for 6 h, using n-hexane; second, silymarin is extracted with methanol for 5 more hours. However, the application of the mentioned procedure as sample preparation prior to the chromatographic analysis of silymarin would hardly be economical. Not only does it last too long but also uses large amounts of toxic solvents and generates too much waste. Researchers, therefore, have been focused on alternative methods of plant sample preparation that allow for elimination of the drawbacks of the traditional approach. The pressurized liquid extraction (PLE) is one of such emerging methods applied in an increasing number of newer analytical studies (13–17), as it presents important advantages over traditional extraction techniques.

PLE allows us to use extractants at elevated pressure and, hence, at temperatures above their boiling point. High temperature increases the rate of analyte diffusion through a cell wall, its solubility into extractant, and decreases the solvent’s viscosity and surface tension. These factors improve the contact of the analytes with the solvent and enhance extraction efficiency (15). The possibility of PLE application for silymarin isolation from milk thistle was mentioned by Benthin et al. (16). However, there are no literature reports on the influence of PLE extraction conditions on the extraction effectiveness of these compounds. (The effect of hot water extraction conditions on the silymarin extraction effectiveness is known from the literature (18.) As PLE is recognized as one of the most effective extraction techniques used for the isolation of biologically active compounds from plants, the question appears whether its application allows for full isolation of silymarin from the thistle fruits in one-step extraction process (without a defatting step).

The present paper discusses the effectiveness of the PLE process applied for silymarin extraction from the defatted and non-defatted fruits of S. marianum L. Gaertner. The effects of solvent type, temperature of the process, duration of static extraction and the number of extraction cycles on the yield of silymarin from the fruits are examined. The temperature and the time effect of defatting by PLE using n-hexane on the change of silymarin yield are also discussed. The PLE results are compared with the data obtained using Soxhlet extraction.
Materials and Methods

Plant material

Dried fruits of *S. marianum* L. Gaertner were purchased from a local pharmacy (Lublin, Poland) in autumn 2011. A sufficiently large representative sample of the plant material (ca. 500 g) was ground and sieved to obtain the particle size of 0.4 mm. Precisely weighed portions of the material were used for extractions.

Materials and reagents

The standardized dry extract of *S. marianum* L. Gaertner and silybin B (with a purity of 98%), applied as standards, was purchased from Sigma-Aldrich, Poland. Acetone, ethyl acetate, phosphoric acid, *n*-hexane (all of them of analytical reagent grade) and methanol (analytical reagent grade and HPLC grade) were purchased from the Polish Chemical Plant (POCh, Gliwice, Poland). Water was purified using a Milli-Q system from Millipore (Millipore, Bedford, MA, USA). Neutral glass, obtained as a gift from local glassworks (fraction 0.4–0.6 mm), was applied as a dispersing agent in the PLE cell.

Pressurized liquid extraction

PLE was performed with a Dionex ASE200 instrument (Dionex Corp., Sunnyvale, CA, USA). The plant material (0.5 g) was mixed with inert material (neutral glass) and placed into a 22-mL stainless steel extraction cell containing filter paper at the bottom. Another circle of filter paper was placed at the top of the extraction cell. Finally, the cell was tightly closed and placed in the heating oven.

The content of the cell was extracted at the operating pressure of 60 bar. At the end of the process, the extracted sample was flushed using the solvent volume equal to 60% of that of the extraction cell. Finally, the sample was purged for 60 s applying pressurized nitrogen (150 psi.), and the extract was collected into a 60-mL glass vial with a Teflon-coated rubber cap. The volume of the collected extract was between 25 and 31 mL, depending on the packing density of the extraction cell. The obtained extract was transferred into a 50-mL volumetric flask and filled up to its volume using an appropriate solvent type. Three independent extractions were performed under the same conditions. Between the runs, the system was washed with an appropriate extraction solvent.

PLE parameters under study were solvent type (methanol, acetone and ethyl acetate), temperature (50, 75, 100, 125 and 150°C), time (5, 10, 15 and 20 min) and the number of extraction cycles (1–5). For the PLE defatting process, *n*-hexane was applied as solvent, and parameters under study were temperature (50 and 100°C) and time (5 and 10 min) of lipids removal. Acetone and ethyl acetate extracts were evaporated to dryness under vacuum and redisolved in methanol before chromatographic analysis.

Soxhlet extraction

Exhaustive extractions in the Soxhlet apparatus were performed using 2.0 g portions of the material. Precisely weighed samples were transferred to a paper thimble. The loaded thimble was inserted into a 100-mL Soxhlet extractor. Extractions were performed in the two-step process (*n* = 3). In the first step of the procedure, the plant material was defatted for 6 h using 75 mL of *n*-hexane. In the second, silymarin was extracted for 5 h with 75 mL of methanol. After cooling to room temperature, the obtained extract was transferred to a 100-mL volumetric flask, which was subsequently filled up to its volume with methanol. Three independent extractions were performed.

Chromatographic analysis of extracts

HPLC measurements were performed on a Dionex Liquid Chromatograph (Dionex Corp.) consisting of a chromatography enclosure (LC20) containing a PEEK automated injection valve equipped with a 10-μL sample loop, a gradient pump (GP50), an absorbance detector (AD25) and a photodiode array detector (PDA100). The whole chromatographic system was under the control of the PeakNet6 data acquisition system. Chromatographic separations were carried out at 40°C using a Prodigy ODS-2 column (5 μm, 250 × 4.6 mm, 1D) (Phenomenex, Torrance, CA, USA). Mobile phase A was a mixture of methanol with aqueous phosphoric acid solution containing 0.5 mL of 75% phosphoric acid in 100 mL of solution (5 : 95, v/v). Mobile phase B was a mixture of methanol with the aqueous phosphoric acid solution containing 0.5 mL of 75% phosphoric acid in 100 mL of solution (50 : 50, v/v). The flow rate was 0.8 mL/min. The analyses were performed in a mobile phase gradient with the percentage of B in A varying as follows: initial concentration, 0%B; 28 min, 100%; 35 min, 100%B; 36 min 0%B. Before the next analysis, the column was equilibrated using the mobile phase containing 0%B for 20 min. Each extract was HPLC-analyzed three times. The wavelength for detecting flavonolignans was set at 288 nm, and the UV-Vis spectra from 210 to 500 nm were also recorded for peak characterization.

The qualitative analysis of the extracts was carried out by comparing the retention times of the peaks and their UV–Vis spectra in the extracts with respect to those of the standardized dry silymarin sample. To prepare the standardized dry silymarin solution, a 0.02-g portion of the sample containing 5.0 mg of silybin A + B was dissolved in 50 mL of methanol. The peaks for silychristin, silydianin, silybin A, silybin B, isosilybin A and B appeared at retention times of 15.1, 17.4, 27.5, 29.1, 33.6 and 34.8 min, respectively. Quantitative analysis was based on silybin B standard, and external standard method was used. A calibration curve was generated from five concentrations of the compound in the concentration range of 0.1–1.0 mg/mL. Three measurements of peak area for each concentration of standard solution were performed. The characteristic parameters of the obtained calibration curve were as follows: slope, 0.516 and intercept, 0.003. The calibration curve was found to be linear in the tested concentration range. The correlation coefficient was found to be >0.995. Because of the difficulty of purchasing silychristin, silydianin, silybin A, isosilybin A and B standards, the amounts of these compounds were calculated by relating their chromatographic responses to the calibration curve for silybin B.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). The analysis of variance (ANOVA) and *t*-test were used to assess the...
influence of PLE conditions on silymarin yield. The mean values were considered significantly different when result of compared parameters differed at \( P = 0.05 \) significance level. To check the significance of each Fisher coefficient, the \( P \)-values were used.

**Results**

Figure 1a presents typical chromatogram of PLE extract obtained from the fruits of *S. marianum* L. Gaertner, whereas Figure 1b shows the chromatogram of silymarin solution prepared dissolving the standardized dry extract of the fruits in methanol (1 mg/mL). The analysis of chromatograms of PLE or Soxhlet extracts with that for standardized solution (retention times, UV–Vis spectra and peak purity index) proved that the applied chromatographic conditions allow for a sufficient resolution of the examined compounds, peaks numbered from 1 to 6, from sample matrix components. The peaks were identified as: (1) silychristin, (2) silydianin, (3) silybin A, (4) silybin B, (5) isosilybin A and (6) isosilybin B, respectively.

The results in Table I present the effect of solvent type (methanol, acetone and ethyl acetate in the case of non-defatted fruits and methanol and acetone in the case of defatted ones) on the cumulative yield of silymarin. The effect of solvent type on the individual silymarin detection is presented in Figure 2. The experiments were performed under a set of preliminary conditions (temperature, 100°C; pressure, 60 bar; static extraction time, 10 min; flush volume, 60%, purge time 60 s and one extraction cycle). Ten minutes PLE at 50°C with \( n \)-hexane was applied to remove lipids from the fruits. Moreover, Table I presents the silymarin yields obtained with methanol, which is the solvent recommended by the European Pharmacopoeia.

The effect of temperature increases on individual components of the silymarin complex extracted from defatted and non-defatted fruits is presented in Table II. The last row of the table contains the total silymarin amount obtained at a given extraction temperature using acetone. The results in Table III present the effect of extraction time on silymarin yields obtained from defatted and non-defatted fruits, using PLE with acetone at 125°C. In this series of experiments, the fruits were defatted by 10 min preliminary PLE at 50°C using \( n \)-hexane. The importance of the experimental factors determined according to the \( F \)-value is listed in Table IV.

Figure 3 presents the influence of various conditions of defatting process on the silymarin yield. To estimate the influence, different extraction temperatures (50 and 100°C) and times (5 and 10 min) of lipids removal, using \( n \)-hexane as solvent, were tested. To isolate the flavonolignans (the second step of the procedure), the same PLE conditions were applied—10 min extraction at 125°C using acetone as solvent. For a better comparison of the impact of defatting process on the silymarin yield, the results presented in Figure 3 are compared with the data obtained, under the same PLE conditions, for the sample not subjected to the process of defatting.

The recovery of silymarin from defatted and non-defatted fruits of *S. marianum* L. Gaertner was determined by consecutive extractions of the same sample under the same PLE conditions (at 125°C for 10 min using acetone, defatting at 50°C for 10 min).

---

**Table I**

<table>
<thead>
<tr>
<th>Amount of silymarin (in mg/g) estimated in milk thistle fruits by</th>
<th>PLE(^a)</th>
<th>Soxhlet(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanol</strong></td>
<td><strong>Acetone</strong></td>
<td><strong>Ethyl acetone</strong></td>
</tr>
<tr>
<td>16.01 ± 1.14</td>
<td>19.10 ± 2.08</td>
<td>10.82 ± 1.12</td>
</tr>
</tbody>
</table>

Data expressed as mean values ± SD (\( n = 3 \)).

\(^a\)PLE conditions: 100°C, 60 bar, 10 min, defatting at 50°C for 10 min.

\(^b\)Soxhlet conditions: the boiling point temperature for 5 h, defatting for 6 h.
**Figure 2.** Influence of extracting solvent type on the PLE yield of individual flavonolignans from milk thistle fruits.

**Table II**

Effect of Extraction Temperature on the Silymarin Yield from the Defatted and Non-Defatted Milk Thistle Fruits Obtained by PLE *

<table>
<thead>
<tr>
<th>Silymarin constituent</th>
<th>Amount of silymarin constituents (in mg/g) obtained at a given temperature from</th>
<th>Defatted material</th>
<th>Non-defatted material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50°C</td>
<td>75°C</td>
<td>100°C</td>
</tr>
<tr>
<td>Silychristin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.50±0.12</td>
<td>2.46±0.18</td>
<td>2.98±0.23</td>
</tr>
<tr>
<td>Silydianin</td>
<td>3.36±0.29</td>
<td>5.66±0.34</td>
<td>7.03±0.31</td>
</tr>
<tr>
<td>Silybin A</td>
<td>1.41±0.12</td>
<td>2.17±0.11</td>
<td>2.54±0.88</td>
</tr>
<tr>
<td>Silybin B</td>
<td>2.32±0.21</td>
<td>3.61±0.28</td>
<td>4.23±0.14</td>
</tr>
<tr>
<td>Isosilybin A</td>
<td>1.01±0.09</td>
<td>1.72±0.13</td>
<td>2.10±0.08</td>
</tr>
<tr>
<td>Isosilybin B</td>
<td>0.53±0.03</td>
<td>0.95±0.09</td>
<td>1.20±0.05</td>
</tr>
<tr>
<td>Total amount</td>
<td>10.18±0.84</td>
<td>16.57±1.07</td>
<td>20.09±0.35</td>
</tr>
</tbody>
</table>

Data expressed as mean values ± SD (n = 3).

*PLE for 10 min with acetone, defatting at 50°C for 10 min.
The extraction of flavonolignans from milk thistle fruits was performed using subcritical water extraction (SWE) and pressurized liquid extraction (PLE) methods. Flavonolignans such as Silychristin, Silydianin, Silymarin, Isosilybin A, and Isosilybin B were isolated. The amounts of these compounds were determined using HPLC analyses. The results are presented in Tables IV and V.

Discussion

Effect of extraction solvent type

The selection of the proper solvent is crucial for obtaining high yields of analytes from plant material. Although in the last decade the application of subcritical water extraction has been reported for the isolation of biologically active compounds from the milk thistle fruits (18–20), it is organic solvents that are most often applied for silymarin extraction.

As results from Table I, the extraction efficiency of acetone is the highest. Methanol extracts a slightly lower amount of the silymarin mixture than acetone, and ethyl acetate isolates the smallest amount of silymarin. The effect of extraction solvent type on the silymarin yield obtained from non-defatted fruits is confirmed by the F-value presented in the last row of Table IV ($F_{exp} > F_{crit}$). It is evident from the results in Figure 2 that the influence of the solvent type on the yield of individual silymarin components is more complex. Acetone and methanol extract comparable amounts of the majority of the investigated flavonolignans. Their amounts are higher than those obtained by means of ethyl acetate. However, in the case of silydianin,

5 min) until no flavonolignans were detected by HPLC. Five independent series of multiple PLE of silymarin were performed. The results are collected in Table V. Moreover, Table V presents the silymarin yield obtained during the recommended extraction procedure in the Soxhlet apparatus. As shown in the table, the extraction in the Soxhlet apparatus gives only ~67% yield of that obtained during multiple PLE and only ~72% of the amount obtained in one-cycle PLE.

Figure 3. Effect of different defatting conditions on the silymarin yield from: (A) sample not subjected to a prior defatting step, (B) sample defatted for 5 min at 50°C, (C) sample defatted for 10 min at 50°C and (D) sample defatted for 10 min at 100°C.

## Table III

Effect of Extraction Time on the Silymarin Yield from the Non-Defatted and Defatted Milk Thistle Fruits Obtained by PLE

<table>
<thead>
<tr>
<th>Silymarin constituent</th>
<th>Amount of silymarin constituents (in mg/g) obtained after a given extraction time (in min) from</th>
<th>Defatted material</th>
<th>Non-defatted material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Isosilybin B</td>
<td>3.68 ± 0.12 3.26 ± 0.07 2.65 ± 0.12 2.39 ± 0.15 3.05 ± 0.09 3.32 ± 0.18 2.96 ± 0.06 2.68 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silydianin</td>
<td>7.43 ± 0.21 6.59 ± 0.19 4.91 ± 0.26 4.66 ± 0.38 6.61 ± 0.12 6.89 ± 0.25 5.84 ± 0.10 4.91 ± 0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silybin A</td>
<td>3.11 ± 0.05 3.03 ± 0.17 2.23 ± 0.05 2.25 ± 0.15 2.39 ± 0.05 3.34 ± 0.17 2.76 ± 0.08 2.28 ± 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silybin B</td>
<td>5.19 ± 0.16 4.72 ± 0.23 3.72 ± 0.09 3.38 ± 0.21 4.51 ± 0.13 5.14 ± 0.19 4.27 ± 0.21 3.63 ± 0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isosilybin A</td>
<td>2.49 ± 0.02 2.36 ± 0.10 1.83 ± 0.04 1.57 ± 0.03 2.11 ± 0.04 2.58 ± 0.11 2.17 ± 0.09 1.94 ± 0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isosilybin B</td>
<td>1.32 ± 0.03 1.31 ± 0.06 0.99 ± 0.02 0.81 ± 0.06 0.99 ± 0.06 1.50 ± 0.11 1.19 ± 0.06 1.04 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total amount</td>
<td>23.22 ± 0.53 21.27 ± 0.61 16.33 ± 0.25 15.08 ± 0.68 19.66 ± 0.49 22.76 ± 0.96 19.19 ± 0.38 16.47 ± 0.87</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean values ± SD (n = 3).

## Table IV

F- and P-Values Obtained During Variance Analysis for the Effects of PLE Conditions on the Silymarin Yield from the Non-Defatted and Defatted Milk Thistle Fruits

<table>
<thead>
<tr>
<th>Silymarin constituent</th>
<th>Effect of solvent type for</th>
<th>Effect of temperature for</th>
<th>Effect of time for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol* Acetone*</td>
<td>Methanol* Acetone*</td>
<td>Methanol* Acetone*</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;exp&lt;/sub&gt; P-value</td>
<td>F&lt;sub&gt;exp&lt;/sub&gt; P-value</td>
<td>F&lt;sub&gt;exp&lt;/sub&gt; P-value</td>
</tr>
<tr>
<td>Silychristin</td>
<td>3.32 0.10 0.15 0.70</td>
<td>72.37 6.3 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>20.56 8.2 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silydianin</td>
<td>5.12 0.10 0.52 0.50</td>
<td>73.36 6.1 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>121.63 2.0 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silybin A</td>
<td>3.44 0.10 0.04 0.90</td>
<td>104.10 2.2 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>46.97 1.9 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silybin B</td>
<td>0.75 0.40 0.02 0.90</td>
<td>394.26 2.3 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>33.93 8.6 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isosilybin A</td>
<td>0.01 0.90 0.02 0.90</td>
<td>69.63 3.4 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>48.29 1.7 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isosilybin B</td>
<td>0.02 0.90 0.01 0.90</td>
<td>141.79 8.9 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>21.63 6.6 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silymarin mixture</td>
<td>8.14 0.10 0.42 0.60</td>
<td>152.84 7.1 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>63.70 4.5 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

F<sub>crit</sub> = 7.71.
F<sub>crit</sub> = 5.14.
F<sub>	ext{mean}</sub> = 3.48.
F<sub>crit</sub> = 4.07.
the main flavonolignan in the silymarin mixture, the yields obtained with acetone are appreciably greater than those obtained with any other solvents. Besides, the yields of the compound obtained with methanol and ethyl acetate are almost the same (within the experimental error). The presented effect of extraction solvent type on the silydianin yield is consistent with the literature data (21). In the cited work, acetone gives the highest silydianin yield for shorter extraction times.

The comparison of the silymarin yields obtained using methanol and acetone, without and with preliminary defatting, supports the conclusion that, in PLE, lipids removal has no essential effect on the yield of the silymarin mixture. The obtained silymarin amounts from fat-free fruits are only slightly higher in comparison with those found for non-defatted material. Yet, the F-value shows that the differences between the yields are statistically insignificant ($F_{\text{exp}} \leq F_{\text{crit}}$, see Table IV). The only advantage of defatting prior to the silymarin extraction by more polar solvents is a slightly greater precision of the analytical method. The extracts obtained from fat-free fruits are more transparent, making the chromatographic analysis easier. Lipids elimination from the sample subjected to the HPLC analysis in the reversed-phase mode undoubtedly prolongs the analytical column lifetime.

In the light of the above, the most powerful extraction solvent for silymarin isolation from the milk thistle fruits is acetone. There is no essential difference in the silymarin amount estimated in non-defatted and defatted fruits. The silymarin yield estimated by long-lasting Soxhlet extraction confirms that defatting process has no significant effect on the yield of these compounds in PLE conditions.

### Effect of extraction temperature

To estimate the optimum temperature for the extraction of flavonolignans from the defatted and non-defatted milk thistle fruits by PLE, the extraction efficiency in the temperature range from 50 to 150°C, using acetone (extraction time, 10 min), was examined. Limiting of the extraction temperature range up to 150°C was due to the fact that at higher temperatures cloudy extracts were obtained in PLE. The presence of sediment may cause undesirable effects, e.g., loss of analytes as a result of adsorption, etc.

As results from Table II, a significant increase of silymarin amount is observed when extraction temperature is increased from 50°C up to ~100–125°C, regardless of whether the fruits were defatted or not (see the last row of Table II). The observed increase is connected with the improvement of PLE efficiency through the increase of silymarin diffusion rate from the matrix to the solvent and through the increase of silymarin solubility in the solvent. Furthermore, temperature increase to 150°C diminishes the silymarin yield. The observed decrease probably results from the thermal degradation of silymarin. It should be noted, however, that the effect is smaller in the case of the non-defatted fruits. The correctness of the hypothesis about the thermal degradation of silymarin is supported by the literature data (19, 22).

Although the thermal degradation of silymarin compounds in the cited work was discovered in subcritical water extraction, the effect of high extraction temperature lowering the extraction efficiency of biologically active compounds from plants, using organic solvents, is well known from other reports (17, 23). A smaller degradation of the silymarin compounds at high temperatures for the non-defatted fruits (see Table II) can be explained by a protective effect of lipids.

Taking the presented data into account, 125°C was selected as optimal temperature for PLE of silymarin from the defatted and non-defatted milk thistle fruits.

### Effect of static extraction time

The efficiency of the PLE process depends on the sample extraction time. For the defatted fruits (see Table III), it is observed that the yield of silymarin is diminished when the extraction time increases from 5 to 20 min. When the extraction was performed for only 5 min, the yields of the silymarin compounds were greater than those obtained in the course of 10 min extraction. In the case of the non-defatted fruits, the increase of extraction time results in a small increase and then gradual decrease of the silymarin yield. The smallest amounts of flavonolignans were obtained during the longest static extraction time of 20 min. This finding supports correctness of the conclusion about the thermal degradation of silymarin and suggests that thermal degradation of flavonolignans occurs even at 125°C; however, it becomes visible for longer extraction times. It cannot be, therefore, excluded that a higher optimum temperature will occur at a shorter extraction time, and a lower optimum temperature may occur with longer extraction times.

The observed differences in the extraction behavior of silymarin from the defatted and non-defatted fruits (see Table III) can be explained by the presence of lipids protecting silymarin from degradation and hindering silymarin diffusion into the extractant. The results presented in ref. (21) confirm that lipids removal helps to release silymarin from the fruits without affecting the release of the individual silymarin constituents. Clearly, smaller the F-values obtained for the non-defatted fruits (see Table IV) also confirm the correctness of the conclusion. The high value of F obtained for the defatted fruits, however, shows that an eventual silymarin loss during the fruit defatting process also cannot be excluded.

### Effect of defatting process

The defatting process of the fruits decreases the silymarin concentration in the plant material (Figure 3). The longer extraction time and the higher lipids extraction temperature the greater silymarin loss ($F_{\text{exp}} = 26.54$, $F_{\text{crit}} = 4.1$). The observed loss of the silymarin yield after the lipids removal is consistent with the research reported previously, in which the loss of other...
polar compounds (toxoids) after preliminary PLE of non-polar ballast substances from yew twigs using n-hexane was found (24). In PLE, the preliminary extraction of ballast substances from plant samples apparently leads to the loss of analytes.

In the light of the obtained results, the optimal PLE conditions for analysis of silymarin in milk thistle fruits are as follows: extraction solvent—acetone; temperature—125°C and time—10 min without the preliminary defatting process.

**Recovery of silymarin during consecutive PLE**

Quantitative isolation of silymarin mixture from the non-defatted milk thistle fruits requires five successive extraction cycles on the same sample, whereas four cycles are required for the defatted fruits. The greater number of cycles for the quantitative extraction of silymarin from the non-defatted material results from the presence of lipids hindering silymarin diffusion.

As shown in Table V, the extraction efficiency of PLE is much higher than that of Soxhlet extraction recommended for silymarin isolation from milk thistle fruits.

**Conclusions**

Due to the high content of lipids in the thistle fruits, European Pharmacopoeia recommends a two-step process of silymarin extraction from the matrix: first, fruits defatting for 6 h, using n-hexane; second, silymarin extraction with methanol for 5 more hours. The obtained results show that PLE is a very effective sample preparation method for silymarin extraction from milk thistle fruits. The PLE yields of silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B in acetone are 3.3, 6.9, 3.3, 5.1, 2.6 and 1.5 mg/g of the non-defatted fruits, respectively. The PLE silymarin yield is higher than that obtained using the Soxhlet apparatus. Moreover, PLE application for silymarin extraction significantly reduces the extraction time and volumes of solvents used. The PLE technique allows for the effective isolation of silymarin mixture in a one-step extraction process (without defatting). The presented data also demonstrate that the elimination of defatting from the PLE extraction of silymarin prevents its loss.

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