

## Gram-Scale Purification of Flavonolignan Diastereoisomers from *Silybum marianum* (Milk Thistle) Extract in Support of Preclinical *in vivo* Studies for Prostate Cancer Chemoprevention

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### **Abstract:**

Extracts of milk thistle (*Silybum marianum*, Asteraceae), termed "silymarin," are used worldwide, primarily for hepatoprotective applications and recently for prostate cancer chemoprevention. Silymarin is a mixture of at least eight compounds, and four major constituents are a group of structurally related flavonolignans: silybin A, silybin B, isosilybin A, and isosilybin B. The initiation of *in vivo* studies to compare the respective preclinical activities of each compound required that gram quantities of these diastereoisomers be prepared. Procedures were developed and optimized to produce multigram-scale quantities of each of these in >97% purity. A hybrid chromatographic precipitative technique was developed, whereby mixtures were chromatographed at high concentrations so as to induce formation of a precipitate in the column fractions, yielding samples that were more enriched in the desired compounds than would be obtained solely by the chromatographic steps alone.

### **Article:**

#### **Introduction**

Milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)] has been used since antiquity, especially for hepatoprotection against alcohol, Amanita mycotoxins, and other xenobiotics [1]. Since 1997, members of our team have explored its cancer chemopreventive properties, especially against models of prostate and other epithelial cancers [2]. Milk thistle extracts influence numerous cellular effectors to inhibit tumor cell proliferation, angiogenesis, invasion, and metastasis [3]. Largely, these studies have examined milk thistle as either a complex mixture of at least eight compounds [4], termed 'silymarin', which is a series of seven structurally-related flavonolignans and the flavonoid, taxifolin [5] (Fig.1), or as an approximately equal mixture of the two most prominent flavonolignans [6], termed silibinin, which consists of the diastereoisomers silybin A and silybin B [5]. Although the chemistry of the constituents in milk thistle has been explored since the late 1950 s (reviewed in [7]), members of our team were the first to isolate and characterize all seven of the flavonolignans [silybin A (1), silybin B (2), isosilybin A (3), isosilybin B (4), silychristin (5), isosilychristin (6), and silydianin (7)] [8]; a similar isolation study focusing on compounds 1-4 was described subsequently [9].

As is well known to this audience, an ongoing question in the examination of herbal drugs is whether the mixture or isolated compounds are responsible for the biological activity of an herb [10]. This can now be tested in milk thistle, since all of the individual components have been isolated on the milligram scale yielding enough material for cell culture studies [8]. In an *in vitro* study, we found that 4 was generally the most potent anti-proliferative compound across three prostate carcinoma cell

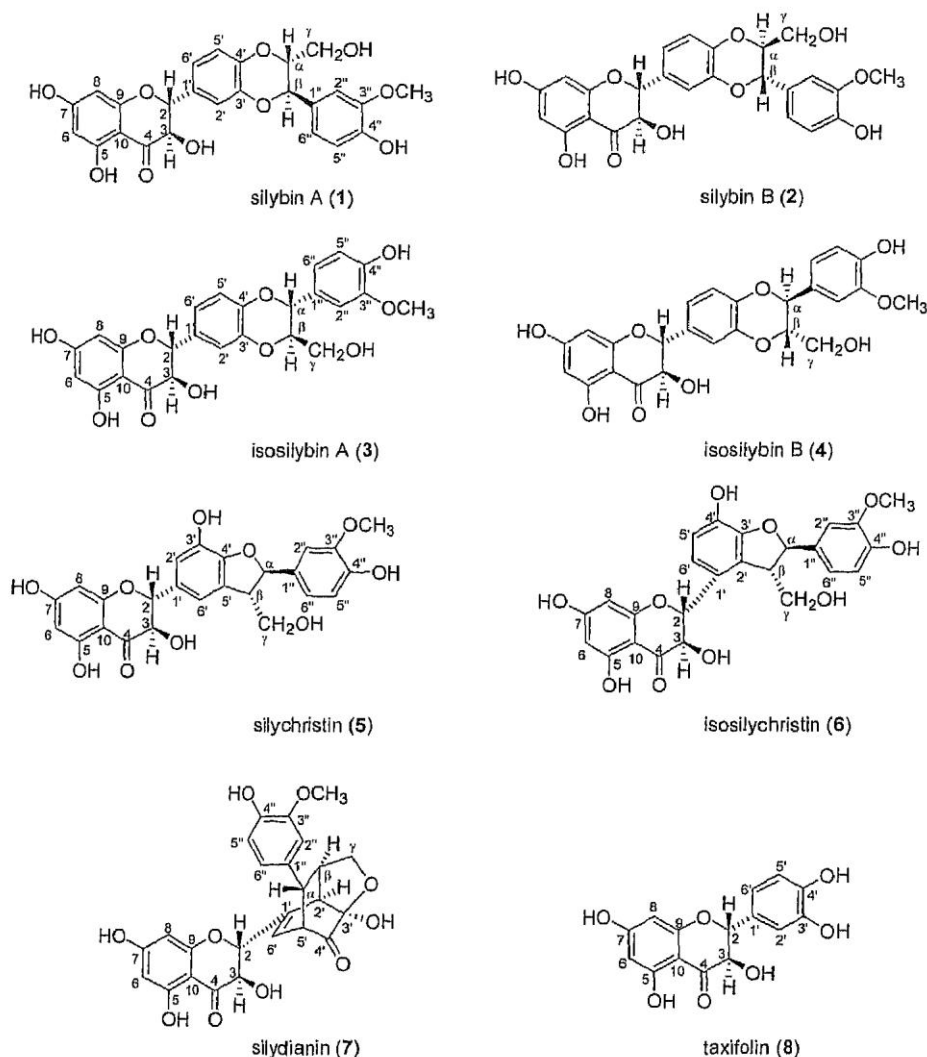


Fig. 1 Structures of the major compounds isolated from milk thistle. An extract that includes all eight compounds is termed 'silymarin', mixtures of only 1 and 2 constitute 'silibinin', and mixtures of only 3 and 4 constitute 'isosilibinin'. These studies focused on isolating gram-scale quantities of 1–4.

lines, but that three other flavonolignans (1-3) each had equal or lesser potency as well [11]. Currently, we are expanding upon those results in a series of mechanistic *in vitro* and *in vivo* models of prostate cancer [12]. A phase I dose-escalation clinical trial with a silibinin-phosphatidylcholine mixture revealed that plasma concentrations of flavonolignans can be achieved in the range where *in vitro* antiproliferative activity has been observed (25 -100  $\mu\text{M}$ ); however, to do so, doses of 5 to 13 g/day were required [13]. To further examine the effects of the flavonolignans in preclinical and clinical settings, both as defined mixtures and as isolated constituents, new methods were necessary to isolate gram-scale quantities of each diastereoisomer. While developing these, several unanticipated empirical observations led to optimized procedures to prepare multigram-scale quantities of compounds 1-4, all in greater than 97% purity.

## Materials and Methods

### Chemicals and instrumentation

HPLC-grade solvents were purchased from Burdick & Jackson (Muskegon, MI, USA). Large-scale separations were accomplished with a CombiFlash Companion flash chromatography system (Teledyne-Isco; Lincoln, NE, USA) using an Isco reverse-phase C-18 column (130 g). HPLC was carried out concurrently on three Varian Prostar HPLC systems (Walnut Creek, CA, USA) equipped with Prostar 210 pumps. Two of these utilized 330 photodiode array detectors (PDA), with data collected and analyzed using Star Chromatography Workstation software (version 5.52). The other system utilized a 335 PDA, with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2). For preparative HPLC, YMC ODS-A (5  $\mu\text{m}$ ; 250x25 mm) columns were used with a 7 mL/min flow rate, while for analytical HPLC, YMC ODS-A (5  $\mu\text{m}$ ; 150x

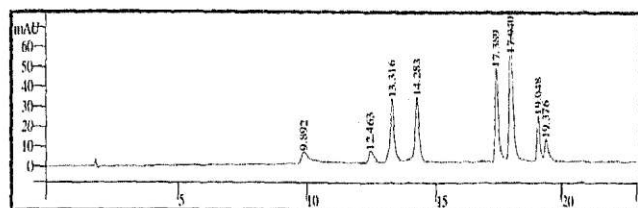
4,6 mm) columns were used with a 1 L/min flow rate (both from Waters; Milford, MA, USA). For analytical HPLC, MetaTherm HPLC column temperature controllers (Varian) maintained these columns at 27 °C.

### **Plant material**

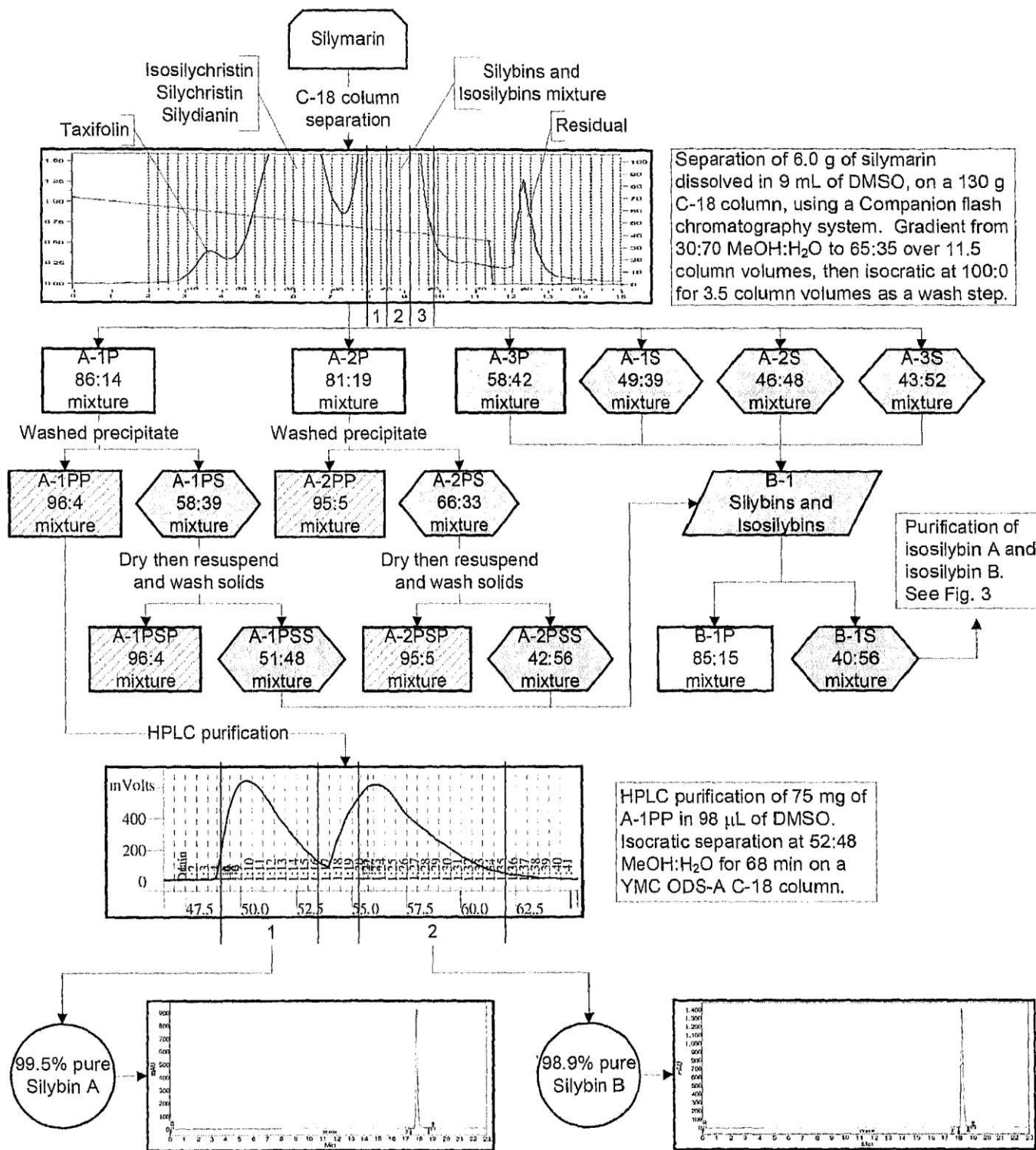
Powdered extract (1 kg; Product No. 345066, Lot No. 37501) of the seeds (achenes) of *Silybum marianum* (L.) Gaertn. was obtained from Euromed, S.A. (Barcelona, Spain), which is a part of the Madaus Group (Cologne, Germany).

### **Analytical HPLC**

The purity of isolates was determined via analytical HPLC using a gradient that started at 30:70 MeOH-H<sub>2</sub>O and increased to 70:30 over 20 min. Chromatograms were observed at 280 nm, and the samples were dissolved in MeOH. The generation and



Analytical HPLC analysis of silymarin extract. Gradient from 30:70 MeOH:H<sub>2</sub>O to 70:30 over 20 min, then isocratic at 30:70 for 5 min.



Figs. 2 and 3 Sequential steps used to isolate gram-scale quantities of compounds 1–4. Squares indicate precipitates; hexagons indicate supernatants; circles indicate pure compounds. Gray shading indicates materials used to generate isosilybinin; diagonal lines indicate materials used to generate pure 1 and 2; horizontal lines indicate materials used to generate pure 3 and 4. In Fig. 2 and the top of Fig. 3, the ratios indicate the relative amounts of silybinin to isosilybinin; on the bottom of Fig. 3, the ratios indicate the relative concentrations of compound 3 (IA) to compound 4 (IB).

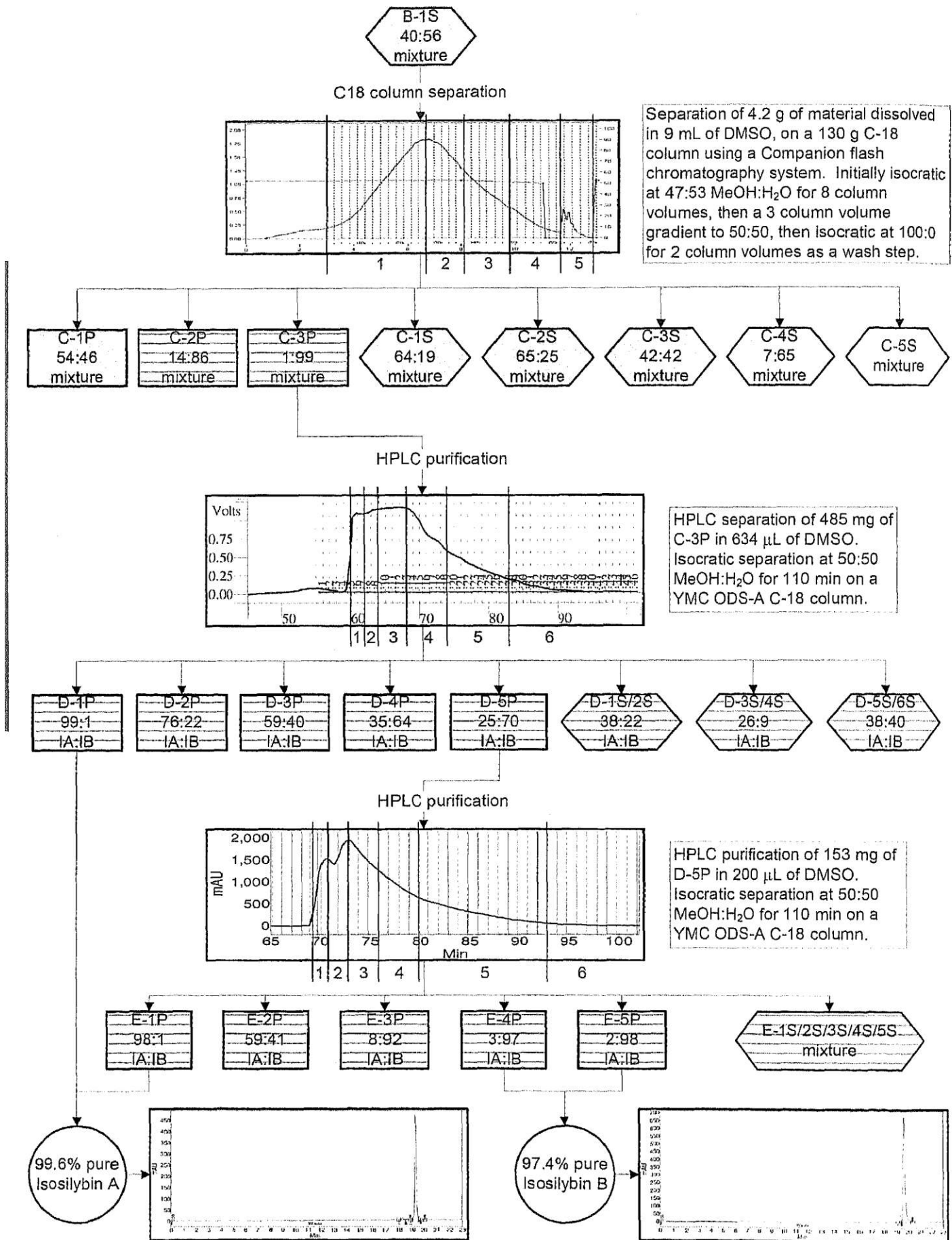


Fig. 3

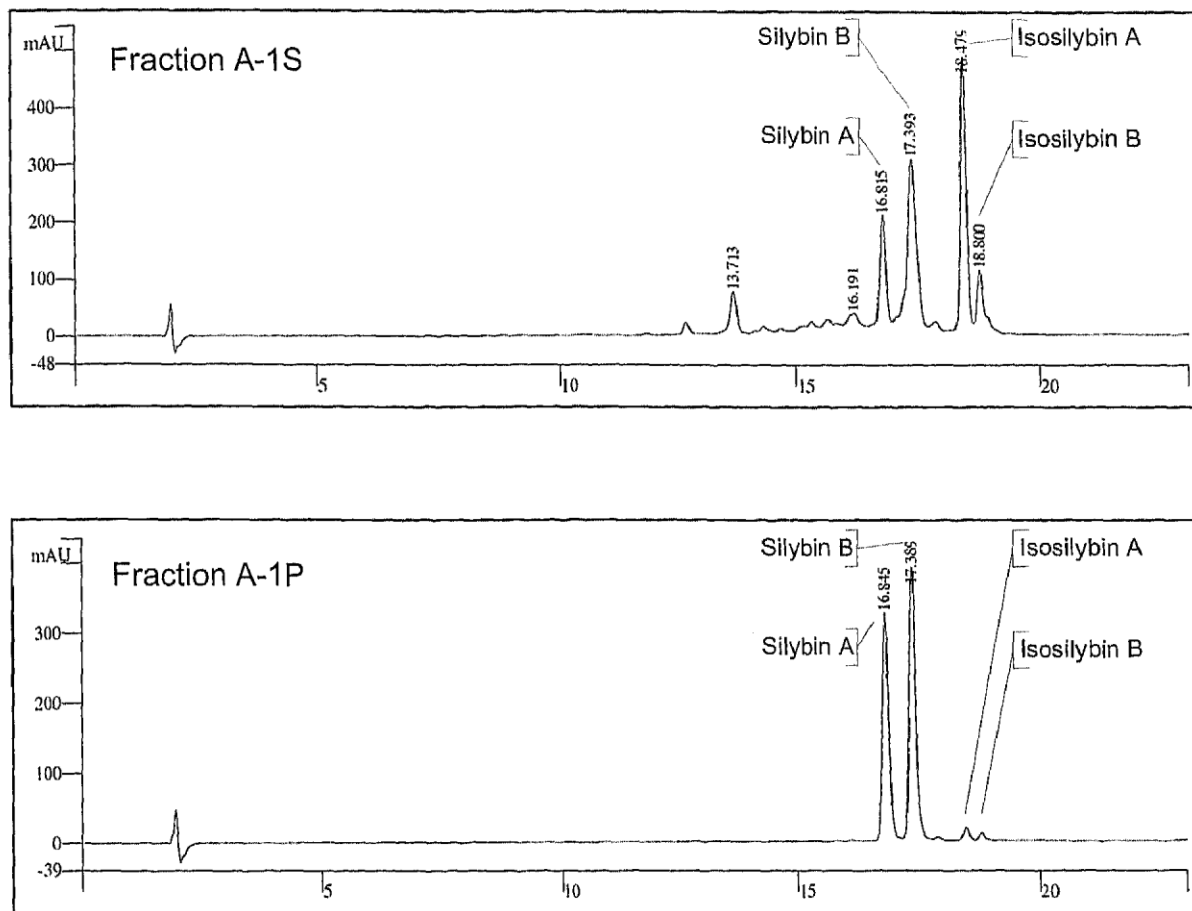


Fig. 4 HPLC traces of supernatants versus precipitates, illustrating the differences between the two types of precipitates that formed depending on the purification step. Fig. 4 compares the supernatant A-1S to the precipitate A-1P; Fig. 5 compares the supernatant C-3S to the supernatant C-3P. Such precipitation steps were critical to the gram-scale isolation of these diastereoisomers.

characterization of reference standards from milk thistle, including the determination of chromatographic retention times, has been described previously [5], [8], [11].

## Results

Milk thistle literature is somewhat confusing, especially in pharmacology studies, due largely to inconsistencies in the nomenclature of extract versus mixtures versus pure compounds. We have reviewed this recently [5] and others have voiced similar concerns [14]. Briefly, in all discussions herein, silymarin refers to the crude mixture of eight compounds shown in Fig. 1, silibinin refers to a roughly equal mixture of 1 and 2, and isosilibinin refers to a roughly equal mixture of 3 and 4.

The top of Fig. 2 displays an analytical scale HPLC chromatogram of the silymarin extract that was used as starting material in the following purifications. As we reported previously, this extract consists of silybin A (1; 16.0%), silybin B (2; 23.8%), isosilybin A (3; 6.4%), isosilybin B (4; 4.4%), silychristin (5; 11.6%), isosilychristin (6; 2.2%), silydianin (7; 16.7%), and taxifolin (8; 1.6%) [11]. To prepare silibinin and isosilibinin, fractionation of powdered silymarin (6.0 g dissolved in 9 mL of DMSO/chromatography for 42 chromatographies) was initiated using the CombiFlash system via a MeOH-H<sub>2</sub>O gradient from 30:70 to 65:35 over 11.5 column volumes (Fig. 2). Precipitates were evident in many fractions upon elution, and fractions from all 42 separations were pooled by their relative ratios of silibinin to isosilibinin to yield ten pooled fractions (A series). These stood for several days (typically 3–5) at room temperature to ensure complete precipitation. Three groups of fractions were filtered separately to obtain precipitates A-1P (40.5 g), A-2P (36.4 g), and A-3P (4.2 g) and supernatants A-1S (7.4 g), A-2S (19.1 g), and A-3S (14.8 g). Precipitates A-1P and A-2P were



washed extensively with 100% MeOH to produce MeOH-insoluble fractions [A-1PP (30.4 g) and A-2PP (24.7 g)], which consisted primarily of silibinin. The corresponding MeOH-soluble fractions [A-1PS (10.1 g) and A-2PS (11.7 g)] were dried, and upon resuspension in MeOH, a significant quantity of silibinin failed to go into solution, was removed by filtration [creating fractions A-1PSP (3.7 g) and A-2PSP (4.3 g)], thereby yielding fractions enriched in isosilibinin, termed A1PSS (2.3g) and A-2PSS (6.5 g) [15]. A series of fractions were combined to form the starting material for the purification of compounds 3 and 4 (fraction B-1), and this was first filtered to remove more MeOH-insoluble silibinin [fraction B-1P (4.4 g)], yielding isosilibinin-enriched fraction B-1 S (50.2 g). In summary, two major fractions were created, fraction A-1PP (silibinin), which was carried forward to generate compounds 1 and 2, and fraction B-1S (isosilibinin), which was carried forward to generate compounds 3 and 4.

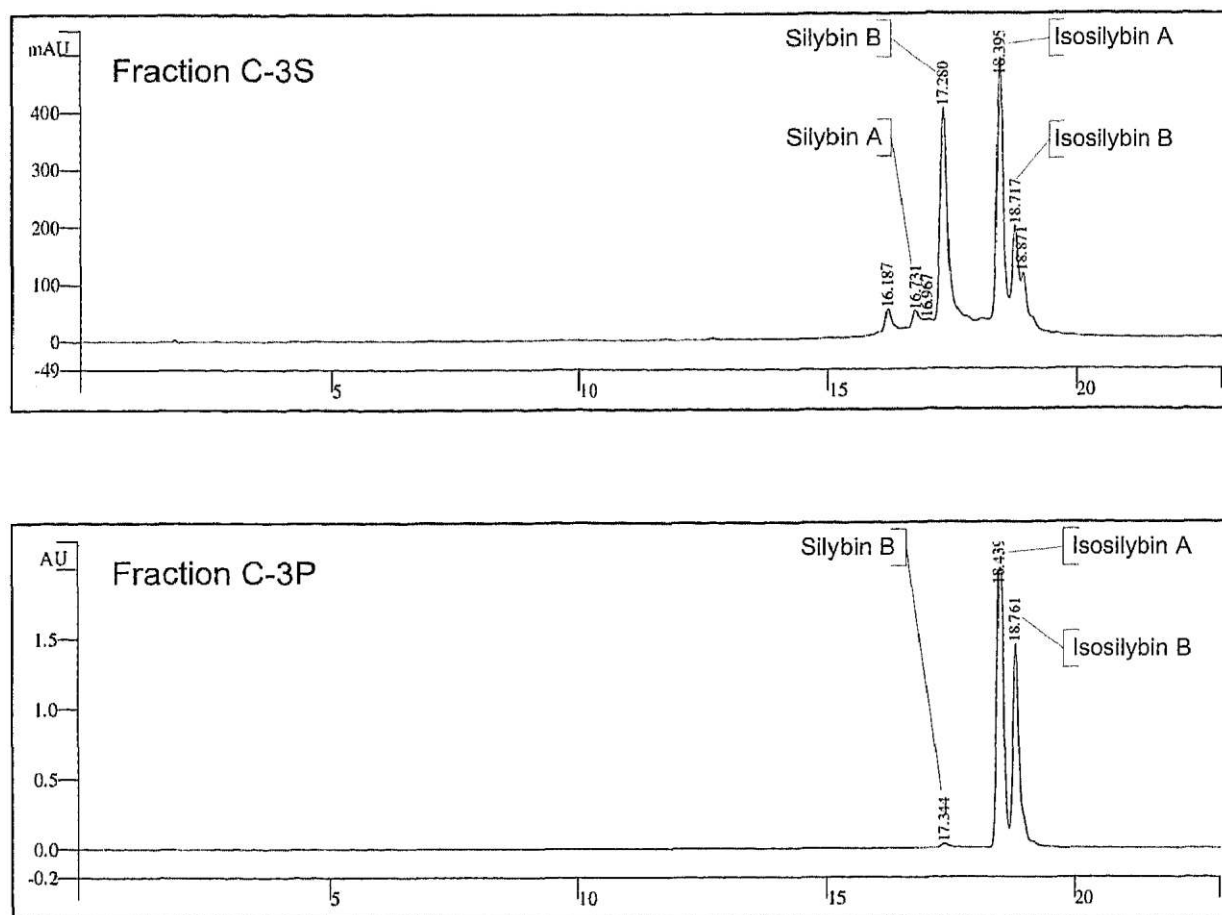


Fig. 5 HPLC traces of supernatants versus precipitates, illustrating the differences between the two types of precipitates that formed depending on the purification step. Fig. 4 compares the supernatant A-1S to the precipitate A-1P; Fig. 5 compares the supernatant C-3S to the supernatant C-3P. Such precipitation steps were critical to the gram-scale isolation of these diastereoisomers.

To prepare silybin A (1) and silybin B (2), approximately 12g of fraction A-1PP were purified via RP-HPLC using an isocratic MeOH-H<sub>2</sub>O system (52:48; Fig.2). Each separation utilized 75 mg of A-1PP dissolved in DMSO (98  $\mu$ L) to give an injection volume of 150 $\mu$ L, and this was repeated 154 times to afford silybin A (1; 4.1 g) and silybin B (2; 4.9 g), each >98% pure.

To prepare isosilybin A (3) and isosilybin B (4), fraction B-1S (50.2 g) was purified over C-18 using MeOH-H<sub>2</sub>O via the CombiFlash system, initiating with an isocratic system (47:53) over 8 column volumes, followed by a gradient to 50:50 over 3 column volumes, and concluding with a wash of 100% MeOH for 2 column volumes. Each separation utilized 4.2 g of B-1S dissolved in 9 mL DMSO, repeating a dozen times to yield ten pooled fractions (C series; Fig. 3). As with the initial separations described above, a key component was the formation of a precipitate, which in this case was enriched with isosilibinin. Fraction C-3P was purified further by RP-

HPLC using an isocratic MeOH-H<sub>2</sub>O system (50 : 50). Each separation utilized 485 mg of C-3P dissolved in DMSO (634  $\mu$ L) to give an injection volume of 970  $\mu$ L, and this was repeated ten times to yield eight pooled fractions of both supernatants and precipitates (D series). Precipitate D-1P (602 mg) afforded isosilybin A (3) in > 99% purity. Fraction D-51<sup>3</sup> (413 mg) and fractions from other C-series fractions further enriched with a similar ratio of isosilybin A:isosilybin B (2652 mg) were purified by RP-HPLC using an isocratic solvent system of MeOH-H<sub>2</sub>O (50:50). Each separation utilized 153 mg dissolved in DMSO (200 L) to give an injection volume of 306 AL, and this was repeated 20 times to yield the E series of fractions, all of which had various ratios of compounds 3:4. In particular, fraction E-11<sup>3</sup> resulted in another aliquot of 3 (285 mg), and fractions E-4P and E-5P were combined to yield isosilybin B (4; 1239 mg; >97% pure). Residual fractions from the C, D, and E series were recycled and purified iteratively in a similar manner to generate in total 4.4g of 3 and 3.7 g of 4, both at >97% purity.

### Discussion

Using these optimized purification procedures, it is possible to generate gram quantities of each diastereoisomer within a few months. This is in contrast to our earlier chemical research on milk thistle, where it took nearly a year to isolate these diastereoisomers on the milligram to hundreds-of-milligram scale [8], [11]. Although a few papers have emerged recently on the chromatography of milk thistle on the analytical scale, especially for quantification purposes [16], [17], [18], [19], to the best of our knowledge, this is the first description of the isolation of these diastereoisomers on the multigram-scale, thereby affording much needed study materials for detailed *in vitro* and *in vivo* pharmacological evaluation.

Of all the diastereoisomers, the rate-limiting step was the gram- scale isolation of isosilybin B (4), and two empirical observations were critical for its eventual purification. First, chromatographic resolution, even during preparative scale flash chromatography, was greatly enhanced by dissolving the injected study sample in a minimum amount of DMSO. As a rough guide, we multiplied the weight of the study material (in mg) by 1.3 to determine the optimum volume of DMSO (in  $\mu$ L). Secondly, using these supersaturated materials, two types of precipitates would form almost immediately upon column elution. In the initial steps (Fig. 2), the precipitate consisted largely of silibinin. Fig. 4 compares the supernatant (A-1S) versus the precipitate (A-1P), and from this, it should be evident why compounds 1 and 2 were isolated readily. We could have isolated many more grams of each of these, but instead, focused on the much more challenging purification of compound 4. In fact, earlier researchers have alluded to recrystallizing silymarin to generate silibinin [20]. Perhaps more importantly, removing as much silibinin as possible via precipitation was critical to the eventual purification of compounds 3 and 4. When the supernatant with low silibinin content was further purified (Fig. 3), new types of supernatant (C-3S) vs precipitate (C-3P) were realized. As shown in Fig. 5, this precipitate was largely isosilybinin, which served readily as starting material for the isolation of pure 3 and 4. In short, supersaturation of starting materials afforded precipitation in column fractions, and in doing so, permitted the rapid isolation of gram-scale quantities of all compounds, especially compound 4.

In conclusion, our collective research goal is to determine the biological effects of milk thistle compounds, both as single entities and defined mixtures, as prostate cancer chemopreventives. To do so, it was critical to develop methods to prepare gram-scale quantities of each diastereoisomer. With these materials in hand, several *in vitro* and *in vivo* studies are progressing. For example, isosilybin A (3) and isosilybin B (4) exert profound growth inhibitory effects in androgen-dependent human prostate carcinoma cells by suppressing regulatory proteins required for entry into the G1 -S phase of the cell cycle [12]. These pure compounds also trigger apoptosis by causing activation of caspase-9 and caspase-3 and suppression of the inhibitor of apoptosis, survivin. While isosilybin B (4) was a slightly more potent growth suppressor, isosilybin A (3) was a more effective apoptosis inducer, and both had minimal effects on non-neoplastic human prostate epithelial cells. These interesting results are being explored using human prostate carcinoma xenografts *in vivo* and are ongoing at the time of publication. Therefore, the availability of the pure compounds is beginning to permit dissection of the chemopreventive and anticancer actions reported previously for the crude mixture silymarin and the partially purified silibinin [3].



## Acknowledgements

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