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Analysis and comparison of active constituents in commercial standardized silymarin extracts by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A sensitive method for the simultaneous quantitation of six active constituents in commercial silymarin standardized extracts was developed based on liquid chromatography (LC) in combination with mass spectrometry (MS). The six main active constituents, namely, silydianin, silychristin, diastereoisomers of silybin (silybin A and B), and diastereoisomers of isosilybin (isosilybin A and B) were completely separated and quantified by LC/MS. Silymarin obtained from Sigma–Aldrich Co. was evaluated and used as standard reference material for the six individual constituents in comparing the relative content of silymarin and the relative ratio of each constituent in commercial standardized silymarin extracts, respectively. Significant variation was found between different commercial silymarin sources. As a result, this method has proven useful in evaluating and quantifying the six active constituents in commercial milk thistle extracts. The calibration curves were over the range from 0.25 to 100 μg/mL for silychristin and silydianin, and from 0.10 to 100 μg/mL for silybin A, silybin B, isosilybin A and isosilybin B, respectively ($r^2 \geq 0.9958$). For all six active constituents, the overall intra-day precision values, based on the relative standard deviation replicate for four QC levels, ranged from 1.18% to 12.4% and accuracy ranged from 89.4% to 112%. This methodology could easily be incorporated into standardized testing to assess content uniformity including lot-to-lot variation as part of routine process controls as well as a means to describe cross-product variation among the exiting marketed formulations.
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Keywords: Silymarin; Milk thistle; Silybin; Isosilybin; LC/MS

1. Introduction

Silymarin, derived from the milk thistle plant *Silybum marianum*, has been used widely for centuries for the protection of the liver from toxic substances. It has also been used for the treatment of toxic liver damage and for the therapy of hepatitis and cirrhosis [1–5]. In addition to its antioxidant properties, it has been reported to have exceptionally high anti-tumor promoting activity [5–9] and has also been linked to the prevention of skin carcinogenesis [10]. Silymarin primarily consists of an isomeric mixture of active flavonolignans: silychristin (Sc), silydianin (Sd), and two groups of diastereoisomeric flavonolignans, silybin A (Sb A) and silybin B (Sb B), and isosilybin A (ISb A) and isosilybin B (ISb B) [11–16]. The different isomers of silymarin have been reported to have different biological activities [17–24]. The chemical structures of the six main active constituents of *Silybum marianum* are shown in Fig. 1.

Standardized *Silybum marianum* contains 70–80% silymarin and has been widely adopted for production. The complexity of the silymarin product combined with its unregulated manufacturing process has made it difficult to judge the role of silymarin in the treatment of chronic liver diseases. This has been further compounded by the poor documentation of its ingredients, its source and its extraction process. As a consequence, the lack of regulation in the manufacturing process has resulted in a great deal of variety in the herbs used for extraction. Herb plants harvested in different geological regions and seasons have been well known for affecting the quantities of chemical components and potentially the efficacy of the extracts [23,25–27]. The quality

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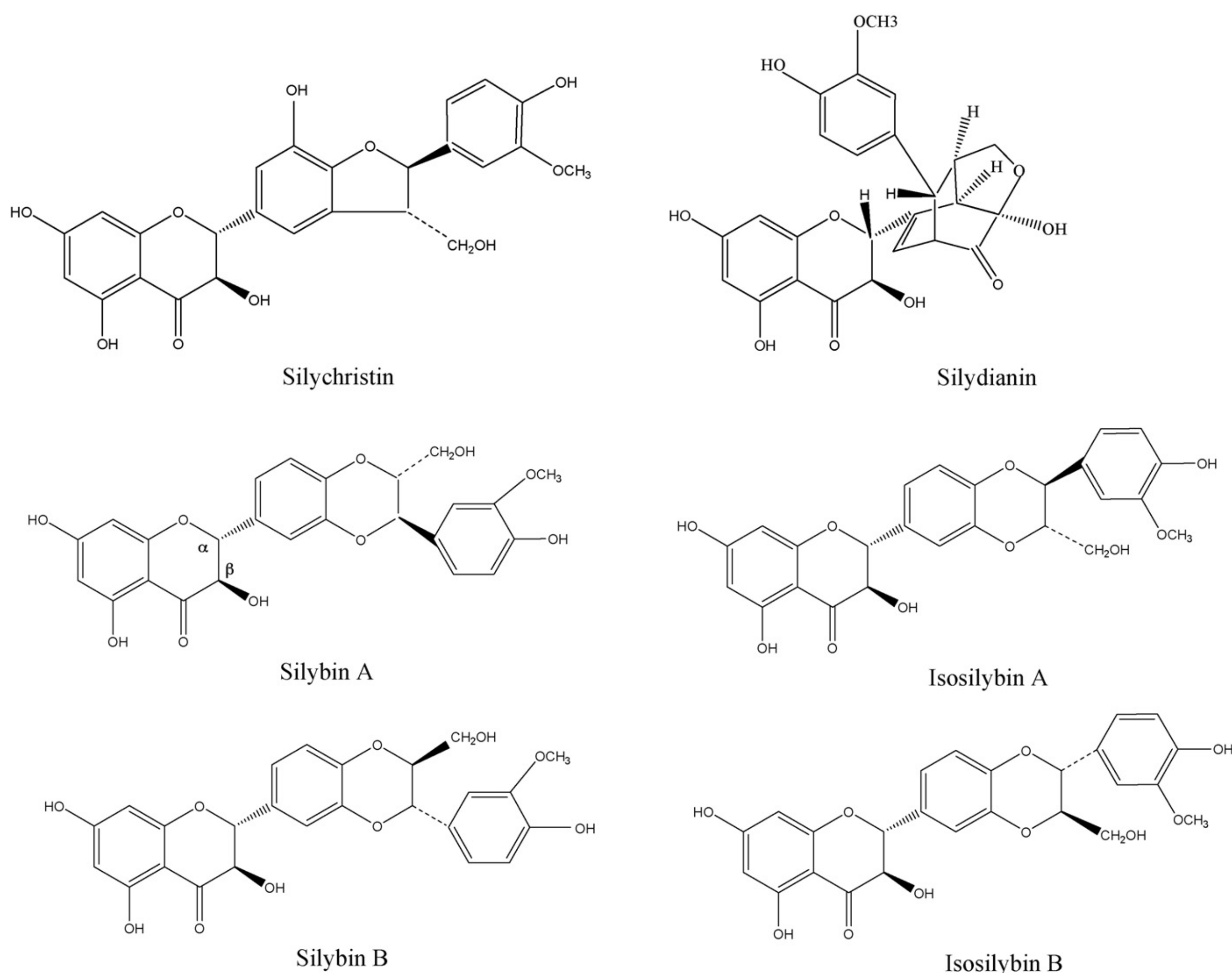

Fig. 1. Structures of the main active constituents in *Silybum marianum*: silychristin, silydianin, diastereomers of silybin (silybin A and B), and diastereomers of isosilybin (isosilybin A and B).

control of the starting material and the final standardized extracts needs to be assessed. Since there have been no criteria or guidelines for the expression of the quality of silymarin extracts, it is difficult to interpret the historical clinical efficacy studies, especially those of varied drug products. Furthermore, many pharmacological studies on silymarin conducted using standardized plant extract have failed to identify the manufacturing source of silymarin and to quantitate the silymarin contents, including its individual active components [24–30], making the evaluation of dose-exposure relationships ambiguous. As a result, the dose-exposure relationships have continued to be poorly defined often representing exposures of mixtures known to have discrete pharmacokinetic properties. Therefore, there is a pressing need for an analytical method that can be used for the quality control of each individual constituent in different silymarin products.

Several chromatographic methods have been reported for the separation or quantitative measurement of individual silymarin. Published methods include those based on thin-layer chromatography (TLC) [31], high-performance liquid chromatography (HPLC) separation with ultraviolet (UV) [15,32–37], column-switching with electrochemical [15], mass spectrometry (MS) [36] or tandem mass spectrometry (MS/MS) [16] detections, and capillary electrophoresis (CE) [37]. Recently, Ding et al. reported an HPLC method that separates all six constituents and is detected by a diode-array detector (DAD) [33]. In the proposed method, silybin and isosilybin were used to quantify the concentrations of silybin (A and B) and isosilybin (A and B) in silymarin, respectively. Moreover, the HPLC-DAD assay was considered to be of insufficient sensitivity, especially for the clinical pharmacokinetic study samples; the standard working ranges for the method are: 0.1398–1.398, 0.0846–0.846, 0.1437–1.437 and 0.0885–0.885 mg/mL for the silychristin, silydianin, silybin (A and B) and isosilybin (A and B), respectively.

We have previously reported on a specific and sensitive liquid chromatography/tandem mass spectroscopy (LC/MS/MS) method to characterize all six active components of silymarin

in either commercial standardized extract or plasma samples [16]. The purpose of this work was undertaken to develop a sensitive and specific LC/MS method to simultaneously quantify and compare the ratio of six constituents of silymarin in commercial standardized extract. This sensitive method will eventually be employed to study the pharmacokinetics of orally-administered silymarin; to discriminate the active constituents in the drug product as well as in the plasma samples collected post dose.

2. Experimental

2.1. Reagents and materials

The milk thistle herbal supplements used were standardized extracts from General Nutrition Corp. (GNC) (Pittsburg, PA, USA), Natural Resource Products (Mission Hills, CA, USA), CVS Pharmacy Inc. (Woonsocket, RI, USA), Safeway Inc. (Pleasanton, CA, USA), Spring Valley Herbs & Natural Foods (Springfield, MO, USA) and Rite Aid Corp. (Harrisburg, PA, USA). These extracts were compared to that of the Yiganlin brand from China (Shanghai Wellconie International Pharmaceutical Trading Co., Ltd., Shanghai, China). Reference standard silymarin and hesperetin (the internal standard) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Silychristin, silydianin and silybin were obtained from ChromaDex Inc. (Santa Ana, CA, USA). Silybin was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Reagent grade formic acid (96%) and ammonium acetate were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Other chemicals and solvents were from Fisher Scientific (Fair Lawn, NJ, USA). De-ionized water was prepared in-house using a Milli-Q water purifying system purchased from Millipore Corp. (Bedford, MA, USA).

2.2. Preparation of stock solutions, standards and quality control samples, and internal standard

A stock solution of silymarin standard was prepared by extracting 50 mg of silymarin (Sigma–Aldrich Inc.), lot No. 7929B, with 5 mL of methanol by vortexing for 30 min at room temperature in a 15 mL (17 mm × 120 mm) polystyrene conical tube. Stock solution of internal standard (hesperetin) was prepared in methanol (1 mg/mL). A 100 ng/mL internal standard was prepared by diluting the stock internal standard solution with methanol. A series of standard working solutions for each constituent was created by further dilution of the silymarin stock solution with methanol as follows: 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 μg/mL for Sc and Sd, respectively; 0.10, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 μg/mL for Sb A, Sb B, ISb A and ISb B, respectively.

Method validation was performed by evaluating intra-assay accuracy and precision of the low, mid and high QC concentrations. The QC samples of 0.3 (Low QC), 3.0 (Mid-1 QC), 25 (Mid-2 QC), and 50 (High QC) μg/mL were prepared separately. The standard working solutions (180 μL) were added to internal standard (20 μL) either for calibration curves or for QC in the validation study. All the solutions were stored at 4 °C and brought to room temperature before use.

2.3. Sample preparation

The commercial milk thistle standardized extracts (50 mg) were extracted with 5 mL of methanol by vortexing for 30 min at room temperature in a 15 mL (17 mm × 120 mm) polystyrene conical tube, and centrifuged at 4000 rpm for 10 min. The organic phases were collected and further diluted with methanol. An aliquot of 5 μL was injected onto HPLC column for LC/MS analysis.

2.4. Chromatography

Chromatography was performed using a Waters 2690 HPLC system with a built-in autosampler (Water Corporation, Milford, MA, USA). HPLC separation was conducted on a YMC ODS-AQ C_{18} column (2.0 mm × 100 mm, 3 μ, 120 Å) (Water Corp., Milford, MA, USA) at 40 °C, with a flow rate of 0.2 mL/min using a gradient mobile phase comprised of 5 mM ammonium acetate adjusted to pH 4.0 with formic acid (A) and methanol/water/formic acid (90:10:0.1, v/v/v) (B). The mobile phase was comprised of a 60:40 mixture of component A to B as the initial condition of each chromatographic run and increased to 65% B in a linear gradient in 25 min and then returned to 40% B for 15 min prior to next injection, instead of 40% B for 5 min which was used in the previous study [16]. The autosampler was maintained at 4 °C. An electronic valve actuator with a Rheodyne selector valve was used to divert the LC flow to waste for the first 4 min to minimize contamination of the MS when no data acquisition was taking place.

2.5. Mass spectrometry

LC/MS analyses were performed on API 4000 tandem mass spectrometer (Sciex, Toronto, Canada) using an electrospray ionization (ESI) source in the negative ion mode and the following conditions: Curtain gas, 10 psi; Gas 1 (nebulizer gas) 32 psi; Gas 2 (heater gas) 0 psi; TurboIonSpray (IS) voltage −4500 V; Source temperature 550 °C; Declustering potential (DP) −56; Entrance potential (EP) −8; and Dwell time 250 ms. For full-scan MS analysis, the spectra were recorded in the range *m/z* 100–1000. Analyst® version 1.4 software (Sciex, Toronto, Canada) was used for the control of equipment, data acquisition and processing.

3. Results and discussion

Currently, all six individual purified standards are not available for the quantification of silymarin although there is a wealth of literature available. Recently, Ding et al. [33] achieved complete separation for the six constituents with UV detection, but the quantification of diastereomers of silybin and isosilybin was performed using a combination of silybin (A and B) and

isosilybin (A and B), respectively. The lack of standards available for the quantification of Sb A, Sb B, ISb A and ISb B, as well as the limit of sensitivity of UV detection lead us in search of alternative standard reference materials and methods of analyses.

Several silymarin products produced from different manufacturers were evaluated for the reference standard materials based on the comparison of their physical properties (such as color, particle size and homogeneity of the powder), as well as the content of silymarin and the level of each active constituent in silymarin via LC/MS/MS [16]. We propose using silymarin obtained from Sigma–Aldrich Co. as the reference standard for the six individual constituents, because of its high purity of silymarin and the similar ratio profile of all six components in the commercial standard silymarin extracts. Six different standard curves of individual constituents generated from the reference standard were used to measure each component in silymarin extract, which was used to evaluate each active constituent in seven commercial products from different brands. The low QC (0.3 μg/mL) prepared from the reference standards was used for the performance verification of the instrument during each run.

3.1. Mass spectrometry

The MS was operated in negative ESI with selected ion monitoring (SIM) acquisition mode. Fig. 2 shows the full scan mass spectra (*m/z* 100–1000) of the silymarin obtained from Sigma–Aldrich Co., which were used as reference standards in the study. The major ions observed were *m/z* 481 for silymarin and *m/z* 301 for hesperetin. The HPLC–SIM of molecular ions (*m/z* 481) was used for selective and quantitative detection of silymarin. It was observed that the fragmentations of the full scan mass spectra, except for the major ion *m/z* 481, were considerably different from the current study and from previous reports [16,38]. It appears that the difference in manufacturing

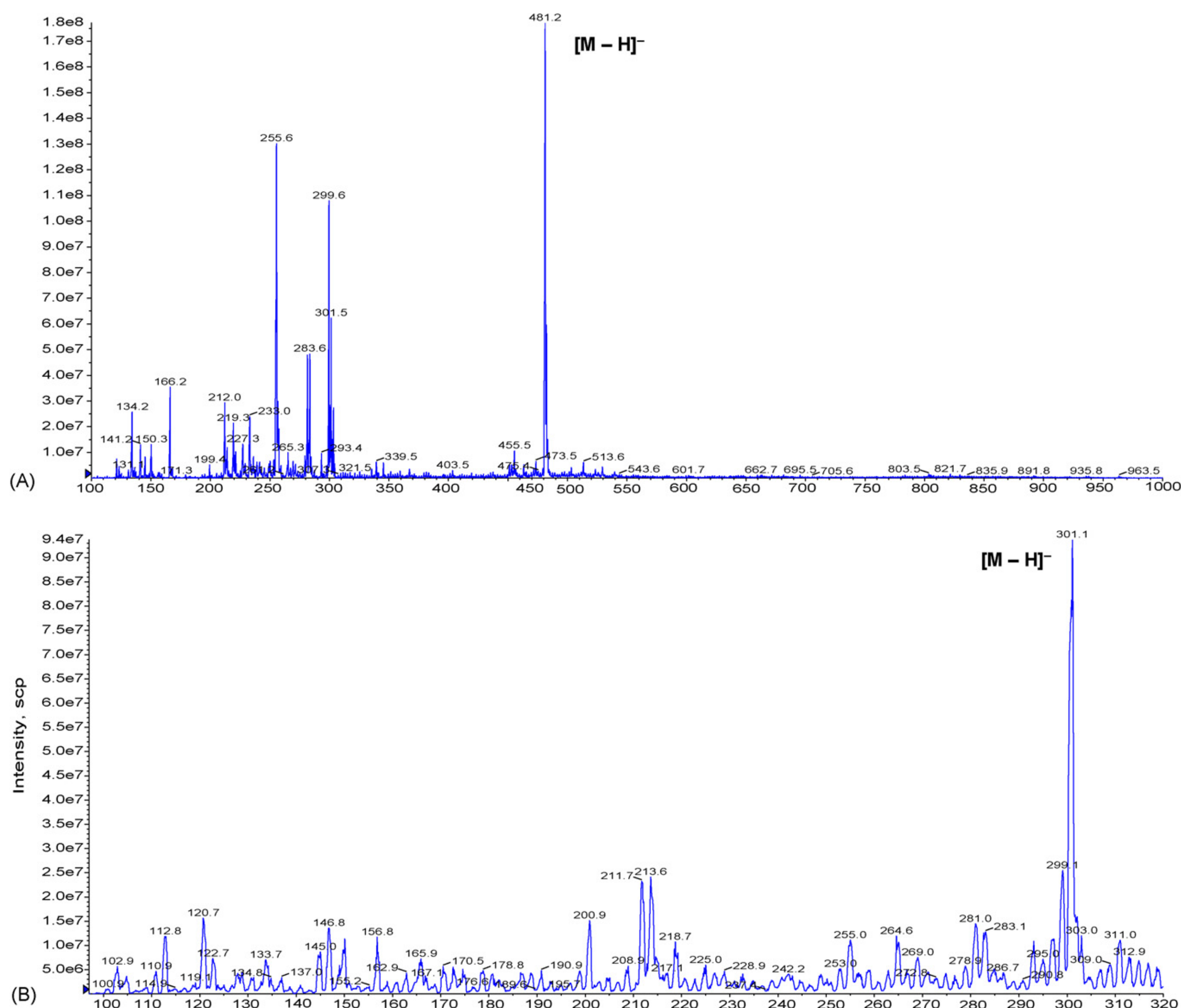

Fig. 2. Q1 full scan mass spectra of (A) silymarin from Sigma–Aldrich Co. and (B) hesperetin (internal standard) by negative TurboIonSpray ionization.

processes causes this difference among the constituents in the silymarin extracts.

3.2. Chromatographic characteristics

The chromatographic profile obtained from the LC-ESI/MS experiment for the $[M–H]^-$ ion at m/z 481 revealed the presence of six major peaks at different retention time (R_t) values. Fig. 3 shows that this analytical method allows for a complete separation with baseline return of the six active constituents and internal standard (hesperetin). The R_t for Sc (1), Sd (2), Sb A (3), Sb B (4), ISb A (5), ISb B (6) were 8.5, 10.0, 15.8, 16.8, 19.7 and 20.5 min, respectively. Two coupled peaks were observed at the retention of Sc. The overlapping peaks hindered the accurate characterization of Sc, although product ion spectra with LC–MS/MS showed a similar pattern. Recently, the Sc isomers, silychristin and isosilychristin, have been isolated using preparative reversed-phase HPLC [9]. Further HPLC separation to resolve and isolate these two peaks was in progress to confirm these two unresolved peaks.

3.3. Calibration curves

The calibration curves were constructed using the ratio of analyte to internal standard peak area (y) against analyte concentrations (x), and the curves were fitted using a quadratic regression model, $y = ax^2 + bx + c$, weighted by $1/x$ in analyst® software, where y is the peak area ratio and x is the concentration of the analyte. The resulting a, b and c parameters were used to determine back-calculated concentrations, which were then statistically evaluated. The standard dynamic range is from 0.25 μg/mL to 100 μg/mL for Sc and Sd and 0.10 μg/mL to 100 μg/mL for Sb A, Sb B, ISb A and ISb B, respectively. The calibration curve range of the method was chosen based on the range of concentrations of each component in silymarin that would be expected in commercial standardized extracts or plasma samples. For all completed experiments to this point, the correlation coefficient (r) for the calibration curves was greater than 0.99. The standard samples were assayed along with QC and unknown samples.

3.4. Sensitivity

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with the coefficient of variation (CV), was ≤20% and the accuracy was within ±20% of the nominal value. As shown in the Fig. 3A, the levels of Sc and Sd were significantly lower than those of Sb A, Sb B, ISb A in the silymarin extract from Sigma–Aldrich Co. The lower limit of quantitation (LLOQ) was determined as 0.25 μg/mL for Sc and Sd in our study. The low sensitivities for Sc and Sd are due to the relative low concentrations of Sc and Sd in reference standard material, and not because of the sensitivity of the LC/MS method. It is not necessary to determine the LLOQ for these four compounds. The standard curves ranging between 0.10 and 100 μg/mL were selected, although the LLOQ can be significantly lower using the detection of the LC/MS technique.

Table 1
Accuracy and precision of QC samples for six active components in silymarin extract

Silychristin ($n = 3$)	0.30	3.00	25.0	50.0
Mean concentration	0.33	3.19	24.1	49.9
Standard deviation	0.03	0.30	1.86	5.88
CV (%)	9.28	10.1	7.46	11.8
Accuracy (%)[a]	109	106	96.6	100
Silydianin ($n = 3$)	0.30	3.00	25.0	50.0
Mean concentration	0.31	3.30	22.3	45.2
Standard deviation	0.03	0.22	0.77	5.87
CV (%)	8.95	7.41	3.08	11.7
Accuracy (%)	104	110	89.4	90.3
Silybin A ($n = 3$)	0.30	3.00	25.0	50.0
Mean concentration	0.30	3.15	23.8	54.2
Standard deviation	0.03	0.14	2.86	6.18
CV (%)	9.83	4.70	11.5	12.4
Accuracy (%)	98.4	105	95.2	108
Silybin B ($n = 3$)	0.30	3.00	25.0	50.0
Mean concentration	0.28	3.32	24.1	46.3
Standard deviation	0.03	0.10	1.15	3.91
CV (%)	10.7	3.21	4.62	7.81
Accuracy (%)	94.7	111	96.2	92.6
Isosilybin A ($n = 3$)	0.30	3.00	25.0	50.0
Mean concentration	0.32	3.20	26.4	50.9
Standard deviation	0.02	0.08	0.30	4.62
CV (%)	7.05	2.73	1.18	9.24
Accuracy (%)	107	107	106	102
Isosilybin B ($n = 3$)	0.30	3.00	25.0	50.0
Mean concentration	0.29	3.36	25.5	51.5
Standard deviation	0.02	0.08	0.36	3.80
CV (%)	7.27	2.76	1.44	7.59
Accuracy (%)	98.0	112	102	103

[a] Accuracy (%) is expressed as (mean found concentration/nominal concentration) × 100%.

3.5. Accuracy and precision

To evaluate the accuracy and precision of the assay, quality control (QC) samples containing Sc, Sd, Sb A, Sb B, ISb A and ISb B at concentrations of 0.3, 3.0, 25 and 50 μg/mL were performed. Intra-day accuracy and precision of the methods were determined by analyzing three replicates of six active components at each of the four concentrations. Table 1 summarizes the means, standard deviation, precision, and accuracy for Sc, Sd, Sb A, Sb B, ISb A, and ISb B at each concentration. Precision was assessed from the % CV of the mean recoveries. As shown in Table 1, the intra-day precision (% CV) over four QC concentrations was 7.46–11.8, 3.08–11.7, 4.70–12.4, 3.21–10.7, 1.18–9.24 and 1.44–7.59% with accuracy range of 96.6–109, 89.4–110, 95.2–108, 92.6–111, 102–107 and 98.0–112% for Sc, Sd, Sb A, Sb B, ISb A, and ISb B, respectively. These data confirm the good precision of the method. The typical chromatograms for low QC, internal standard and blank are shown in Fig. 3.

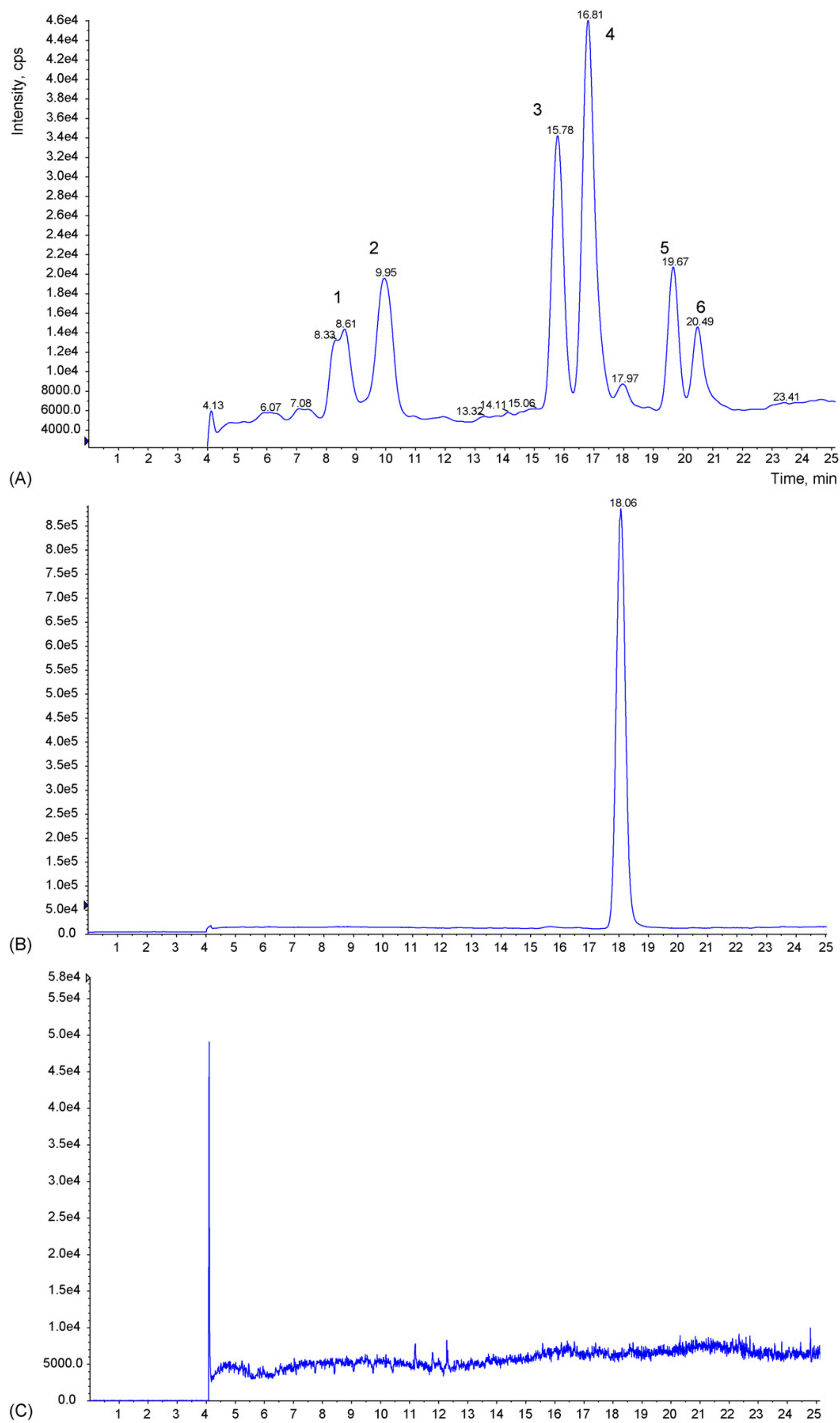

Fig. 3. Chromatograms of (A) low QC [m/z = 481], (B) corresponding internal standard [m/z = 301] and (C) blank [m/z = 481]. Peak identity: 1, silychristin; 2, silydianin; 3, silybin A; 4, silybin B; 5, isosilybin A; 6, isosilybin B.

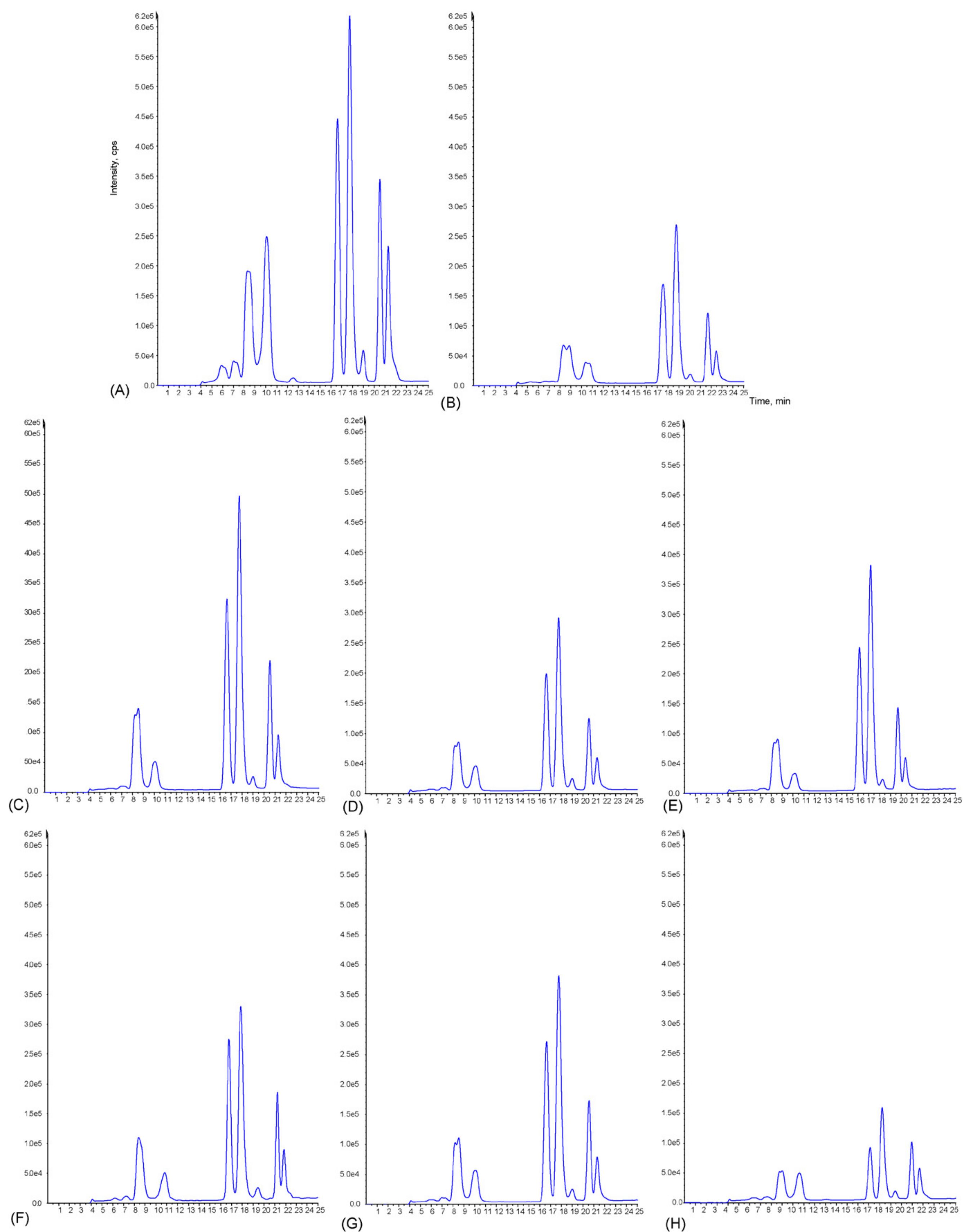

Fig. 4. Chromatograms of the various silymarin samples: (A) Sigma–Aldrich Co. (reference standard for comparison); (B) Safeway Inc.; (C) Natural Resource Product; (D) General Nutrition Corp.; (E) CVS Pharmacy; (F) Rite Aid; (G) Spring Valley; (H) commercially available silymarin sample from China (Yiganlin) (*Note:* All the chromatograms are in the same scale with a maximum intensity and time of 6.2×10^5 cps and 25 min).

Table 2
Quantitative comparison of the constituents of the various commercial silymarin samples in relation to the standard silymarin obtained from Sigma–Aldrich Co

% (μg/mL)	Silychristin	Silydianin	Silibin A	Silibin B	Isosilybin A	Isosilybin B
Sigma–Aldrich	100 (7.73)	100 (7.72)	100 (6.68)	100 (8.51)	100 (7.76)	100 (5.75)
Safeway	44.5[a] (3.68)[b]	15.8 (1.22)	39.7 (2.65)	38.3 (3.26)	37.8 (2.93)	19.7 (1.13)
Natural resource	74.9 (5.79)	15.4 (1.19)	71.6 (4.78)	82.5 (7.02)	59.0 (4.58)	36.7 (2.11)
GNC	47.6 (3.44)	16.6 (1.28)	42.7 (2.85)	44.7 (3.80)	35.4 (2.75)	21.7 (1.25)
CVS pharmacy	59.8 (4.62)	17.6 (1.36)	59.8 (3.94)	62.5 (5.32)	42.3 (3.28)	28.2 (1.70)
Rite aid	54.6 (4.22)	17.2 (1.33)	58.8 (3.93)	53.5 (4.55)	46.3 (3.59)	35.5 (1.97)
Spring valley	55.5 (4.29)	15.4 (1.19)	58.7 (3.92)	60.3 (4.57)	47.6 (3.69)	36.2 (2.04)
Yiganlin	27.2 (2.10)	15.7 (1.21)	22.3 (1.49)	23.6 (2.01)	25.3 (1.96)	21.6 (1.24)

[a] For each constituent, concentartion relative to Sigma–Aldrich reference standard (%).
[b] For each constituent, concentartion relative to Sigma–Aldrich reference standard (μg/mL).

Table 3
Ratio of peak areas of each of the six constituents with respect to total area per commercial sample

% Mass	Silychristin	Silydianin	Silibin A	Silibin B	Isosilybin A	Isosilybin B
Sigma–Aldrich	100	100	100	100	100	100
Safeway	44.5	15.8	39.7	38.3	37.8	19.7
Natural resource	74.9	15.4	71.6	82.5	59.0	36.7
GNC	47.6	16.6	42.7	44.7	35.4	21.7
CVS pharmacy	59.8	17.6	59.8	62.5	42.3	28.2
Rite aid	54.6	17.2	58.8	53.5	46.3	35.5
Spring valley	55.5	15.4	58.7	60.3	47.6	36.2
Yiganlin	27.2	15.7	22.3	23.6	25.3	21.6

3.6. Autosampler stability

Autosampler stability was studied by comparing freshly injected samples with re-injected samples 24 h later. In these experiments, the Low QC, Mid-1 QC, Mid-2 QC, and High QC samples were assayed in triplicate. Results showed that six active components in silymarin remained stable over 24 h in autosampler tray at 4 °C. The accuracy are greater than 92% overall upon re-injection for six components.

3.7. Applications

This method has been applied successfully for the quantitative analysis of the six constituents in the six different silymarin extracts from the United States and also in the Yiganlin silymarin extract from China, all of which were purchased locally in Philadelphia, PA, USA. The constituents of silymarin standardized extracts were identified by comparison of R_t values with those of the reference peaks from silymarin obtained from Sigma–Aldrich Co. Fig. 4 displays the chromatograms of the silymarin extracts generated from different manufacturers. It is quite evident that the chromatograms of the local manufacturers (Figures B–G) differ vastly from that of the Chinese manufacturer (Figure H). Figure A is the reference standard, which is used for comparison purposes. The results of the quantitative analyses are summarized in Table 2, which contains the assay results of the ratios of the concentration of each of the six active constituents expressed the percentage of various commercial silymarin samples in relation to the reference standard silymarin obtained from Sigma–Aldrich Co. The heterogeneity of the various commercial samples is quite evident. More importantly, the Yiganlin silymarin, produced from a Chinese manufacturer, showed significantly lower content of silymarin compared to the other silymarin commercial products tested. Table 3 specifically shows the ratio of each constituent's individual peak area to the total area of all the individual constituents for each commercial sample for each of the six constituents. Although Yiganlin has the lower content of Sb A and Sb B, it has remarkably higher peak area ratios for Sd, ISb A, and ISb B, respectively. Similarly, silymarin from Sigma–Aldrich Co. only shows the higher ratios of Sd and ISb B, respectively. The other six silymarin extracts obtained from the US showed similar ratios for all six individual constituents.

4. Conclusions

A sensitive LC/MS method was developed for the simultaneous determination of six active isomeric flavonolignans in silymarin. The established method has been successfully applied to the identification, quantification and comparison of the active components of silymarin in six commercial products. Silymarin contents varied with respect to different brands of commercial standardized extracts; the ratios of individual constituents were also different. We therefore conclude that silymarin has a varied content and therefore a complex chemical mixture due to its diverse geological origins and/or its different manufacturing processes. Both results strongly indicate that sensitive and specific analytical procedures need to be implemented for quality control of raw material, standardized extracts and manufacturing processes to ensure the quality and consistency of the commercial products. This method has proved to be useful in evaluating and quantifying the six active constituents in commercial milk

thistle extracts and will be applied to the investigation of patients with Hepatitis C [39].

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