

A Validated UHPLC-Tandem Mass Spectrometry Method for Quantitative Analysis of Flavonolignans in Milk Thistle (*Silybum marianum*) Extracts

By: [Tyler N. Graf](#), [Nadja B. Cech](#), Stephen J. Polyak, and [Nicholas H. Oberlies](#)

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Abstract:

Validated methods are needed for the analysis of natural product secondary metabolites. These methods are particularly important to translate in vitro observations to in vivo studies. Herein, a method is reported for the analysis of the key secondary metabolites, a series of flavonolignans and a flavonoid, from an extract prepared from the seeds of milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)]. This report represents the first UHPLC MS-MS method validated for quantitative analysis of these compounds. The method takes advantage of the excellent resolution achievable with UHPLC to provide a complete analysis in less than 7 min. The method is validated using both UV and MS detectors, making it applicable in laboratories with different types of analytical instrumentation available. Lower limits of quantitation achieved with this method range from 0.0400 μM to 0.160 μM with UV and from 0.0800 μM to 0.160 μM with MS. The new method is employed to evaluate variability in constituent composition in various commercial *S. marianum* extracts, and to show that storage of the milk thistle compounds in DMSO leads to degradation.

Keywords: Milk thistle | *Silybum marianum* | Flavonolignans | Silymarin | Validation

Article:

1. Introduction

The medicinal herb, milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)], has been used since antiquity, particularly for hepatoprotective applications [1], and more recently, for prostate cancer chemoprevention [2]. In the modern herbal pharmacopeia, there are two main milk thistle preparations [3]. Silymarin is an extract of the seeds (achenes) and consists of the flavonoid taxifolin (**1**) and at least seven flavonolignans [isosilychristin (**2**), silychristin (**3**), silydianin (**4**), silybin A (**5**), silybin B (**6**), isosilybin A (**7**), and isosilybin B (**8**); Fig. 1], along with other minor

constituents. This extract can be partially purified to form the other main preparation, termed either silybin or silibinin, which is largely a 1:1 mixture of **5** and **6**.

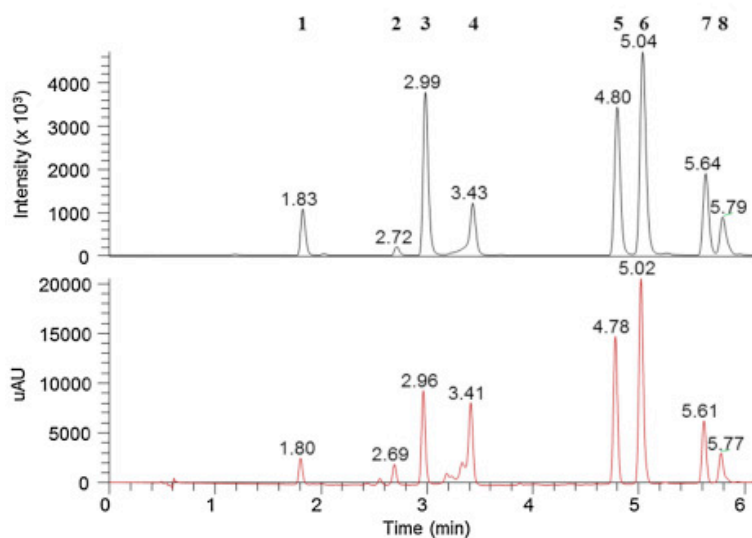
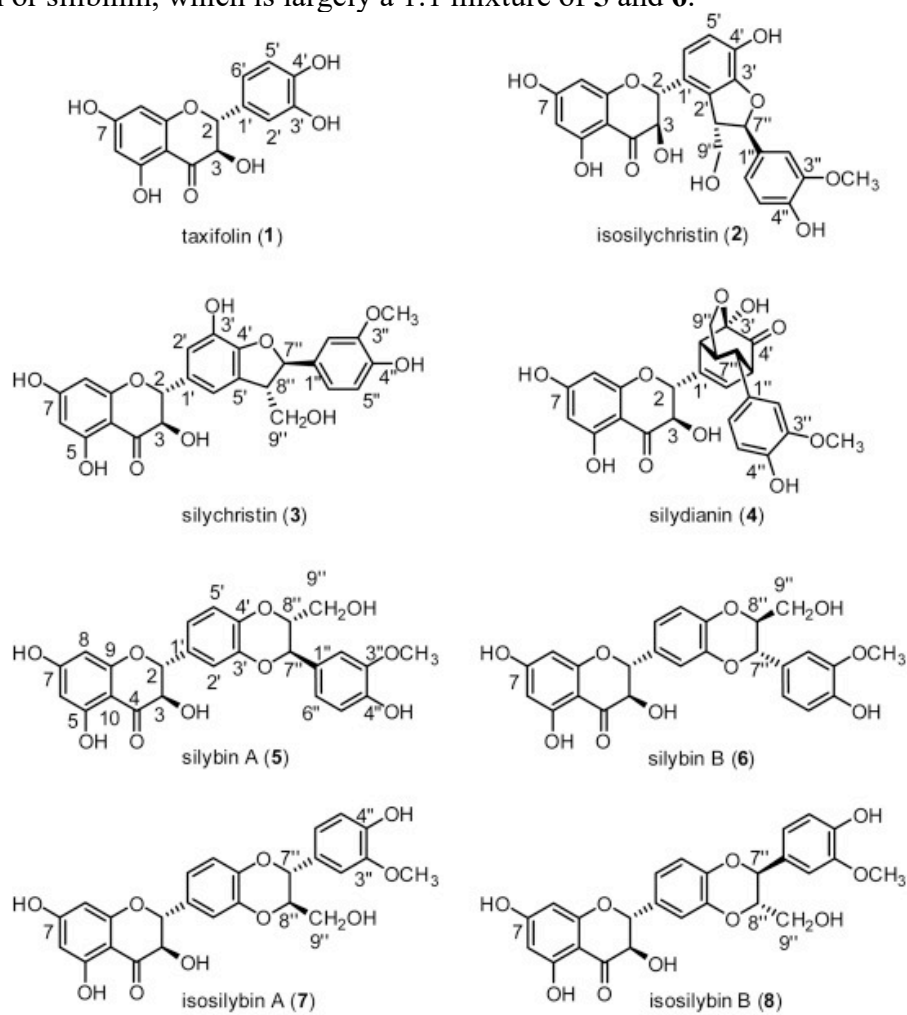


Fig. 1. Structures of compounds **1-8**. The numerical order of the compounds corresponds to their elution order in the MS and UV chromatograms for silymarin extract Madaus 37501.

After over a decade of studying the chemistry of flavonolignans from milk thistle, methods have been developed to separate and identify the flavonolignan diastereoisomers on the gram scale [4]. This supply, in turn, has assisted with the examination of the cytoprotective properties of the milk thistle compounds, where they have demonstrated activity in inhibiting virus infection, preventing oxidative stress, and modulating cellular metabolic and inflammatory status [5], [6], [7]. For effective studies of the biological activity of milk thistle preparations, both in vitro and in vivo, knowledge of the quantity and identity of bioactive constituents in study material is needed. To address this need, the purpose of this study was to develop a validated method for the quantitative determination of milk thistle compounds in extracts and preparations.

In the past few years, there have been three studies devoted to the chemical characterization of milk thistle products. Li et al. [8] developed a 40 min HPLC–MS method for analysis of silibinin (a 1:1 mixture of **5** and **6**) in plasma. Hadad et al. [9] developed an 8 min HPLC method (employing monolithic columns) for analysis of seven compounds from silymarin. The compounds were detected by relying on their absorbance at 288 nm. Finally, Wang et al. [10] employed UHPLC coupled to electrospray ionization mass spectrometry (ESI–MS) for identification of seven compounds from silymarin. Similar to the approach employed by Wang et al. [10], the method described herein relies on UHPLC ESI–MS. A major advantage of this approach is the rapid analysis times that it facilitates (in this case, 6 min). However, unlike the previously published UHPLC ESI–MS method [10], our method is extended to include both quantitation and identification. Indeed, herein is the first validated method for quantitative analysis of all eight major bioactive components of silymarin (**1-8**).

2. Experimental

2.1. Materials and chemicals

The seven flavonolignans and taxifolin (Fig. 1) were isolated as described previously [4], and were all of a high purity ($\geq 98\%$) as determined by UHPLC-UV analysis. Constituent levels were quantified in a number of different silymarin extracts using the methods described herein. Two separate batches of silymarin (product number 345066, lot numbers 37501 and 286061) from Euromed, S.A. (Barcelona, Spain), which is a part of the Madaus Group (Cologne, Germany), a batch of silymarin from Indena S.p.A. (Milan, Italy) (lot number 27691; the source for the isolation of the pure compounds), and two separate batches of silymarin from Sigma-Aldrich (St. Louis, MO, USA) (product numbers S0292 and 254924; lot numbers BCBJ0393V and 05503 PG) were analyzed. Caffeine, HPLC grade acetonitrile, and mass spectrometry grade formic acid, methanol (MeOH), and water (H₂O) were purchased from Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of samples and standards

All compounds were dissolved separately in 1:1CH₃CN:H₂O (with 655 μ M caffeine as internal control) to produce 5.24 mM stock solutions. An equimolar master mixture of the eight analytes was prepared at concentrations of 655 μ M for each individual component (and 655 μ M caffeine). This master mixture was serially diluted 2-fold to produce 17 standard mixtures ranging in

concentration from 328 μM to 0.005 μM . A separate series of quality control samples (QC) was prepared by separately diluting the 655 μM master mixture to 16.4 μM , 1.64 μM , and 0.164 μM (high, medium, and low QCs). A series of silymarin mixtures was prepared in 1:1 $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ at 2.53 mg/mL, and diluted 100-fold prior to analysis.

2.3. UHPLC-UV-MS quantitative analysis

UHPLC analyses were conducted utilizing a Waters Acquity UHPLC system (Milford, MA, USA) equipped with an autosampler, photodiode array detector (PDA), column manager, and binary solvent manager. An HSS-T3 C18 column (100 mm \times 2.1 mm i.d., 1.8 μm packing, from Waters) operated at 50 $^\circ\text{C}$ was used for all chromatographic analyses. The gradient system consisted of 0.1% formic acid in MeOH (B) and 0.1% formic acid in H_2O (A), at a flow rate of 0.5 mL/min. A gradient from 30 to 55% B over 6.0 min followed by reequilibration at 30% B for 0.6 min was used. All samples and standards were analyzed in triplicate using 3 μL injections, with the exception of the QC samples, which were injected in quintuplicate.

The UHPLC system was coupled to a Thermo Scientific TSQ Quantum Access triple quadrupole mass spectrometer (Waltham, MA, USA) with a heated electrospray ionization (HESI) source. Analyses were conducted in the positive ion mode, with a spray voltage of 3800 V, vaporizer and capillary temperatures of 360 $^\circ\text{C}$ and 380 $^\circ\text{C}$, respectively, and sheath gas and auxiliary gas of 50 and 45 (arbitrary units), respectively. Tube lens offset and skimmer offset were 122 V and 0 V, respectively.

Quantitative analysis was performed using UV data collected at 288 nm and multiple reaction monitoring (MRM) on the mass spectrometer (MS). The UV and MS data were collected simultaneously for each injection. MRM transitions employed for each compound are given in Table 1. For each analyte, three additional MRM transitions were observed to verify peak identity, and a table of the transitions observed during each of the acquisition segments is provided (Table S1). UV and MS data were collected and analyzed using Xcaliber software (version 2.2, from Thermo Scientific). Peak detection and peak areas were determined using the ICIS algorithm for MS data and the Avalon algorithm for UV data. All calibration curves were generated in Xcaliber using 1/X weighting to create linear curve fits that emphasize the lower concentration calibration points. External calibration was employed for this analysis.

Table 1. Multiple reaction monitoring transitions observed.^a

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Caffeine	1.19	195.1 $[\text{M}+\text{H}]^+$	138.2	22
1	1.82	305.1 $[\text{M}+\text{H}]^+$	259.1	13
2	2.70	483.1 $[\text{M}+\text{H}]^+$	153.0	22
3	2.97	453.1 $[\text{M}-\text{CH}_2\text{OH} + \text{H}]^+$	435.1	12
4	3.42	483.1 $[\text{M}+\text{H}]^+$	153.0	30
5	4.79	483.1 $[\text{M}+\text{H}]^+$	465.1	10
6	5.03	483.1 $[\text{M}+\text{H}]^+$	465.1	10
7	5.63	483.1 $[\text{M}+\text{H}]^+$	329.0	10
8	5.79	483.1 $[\text{M}+\text{H}]^+$	329.0	10

^aThe dwell time for all transitions was 0.040 min.

2.4. Method validation

The identities of the standards were confirmed by NMR and mass spectrometry analyses [4], [11], [12], as well as by comparison of elution time with other identified standards. Linearity of the calibration curves was assessed by least-squares analysis. Precision and accuracy were determined by calculating the relative standard deviation (RSD) and relative error (RE), respectively, for replicate injections. For the purpose of this study, RE is defined as the percent difference between the mean measured concentration of three replicate injections of each standard concentration, and the nominal concentration of that standard. All analyses were performed in triplicate on three different days. Repeatability was evaluated based on the RSD and RE for triplicate analyses in a single day, while intermediate precision was determined based on the interday RSD and RE. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of a given analyte that could be measured with an intraday precision below 15%. For the MS data analysis, the upper limit of quantitation (ULOQ) was defined as the highest concentration where the relationship between MS signal and concentration was linear (as evident by $R^2 > 0.9995$). For the UV data analysis, the ULOQ was set at the highest concentration measured in this study, as the upper limit for linearity in UV signals was not reached. All standard curves were plotted using standard concentrations between, and including, the LLOQ and the ULOQ. The linear dynamic range for each analyte was defined as being all concentrations between, and including, the LLOQ and the ULOQ. The linearity of all of the UV standard curves was verified by each having an $R^2 > 0.9999$.

Sets of high, medium, and low QC samples were placed at the beginning, middle, and end of the sample sets for each day of analysis, and the intraday and interday results for RE and RSD were measured to verify method and instrument stability across the days of analysis. Caffeine was included as an internal control in all standard and QC samples to monitor consistency in retention times and linearity of both dilutions and instrument response.

2.5. Matrix effects

Matrix effects were examined by comparison of quantitation results for silymarin samples spiked with blank solvent versus the results for the same samples spiked with an equal volume of a solution containing all eight of the analytes. Matrix effects were determined by subtracting the area for the analyte of interest in the unspiked extract (A_{matrix}) from the area in the spiked extract ($A_{\text{matrix,spiked}}$), and dividing by the area for the analyte in solvent without matrix (A_{solvent}) (Eq. (1)).

$$\%ME = 100 \times \frac{A_{\text{matrix,spiked}} - A_{\text{matrix}}}{A_{\text{solvent}}} \quad (1)$$

2.6. Evaluation of compound stability

UHPLC analyses were completed utilizing a Waters Acquity UHPLC system similar to the one described in Section 2.3. All degraded samples used for this experiment were from the same batches of the eight compounds as were used for the validation study. Degraded samples were prepared at 500 μM in DMSO from pure (non-degraded) dry stocks of each analyte and stored at room temperature for 6 months (191 days) before analysis. The fresh samples of each compound

were prepared in MeOH from the same dry stocks of the compounds (stored dry at 4°C over the same 6 months) immediately before analysis.

3. Results and discussion

3.1. Method validation

Calibration curves for all standards exhibited coefficients of determination (R^2) greater than 0.9995 for MS data (Table 2) and 0.9999 for UV data (Table 3). Baseline resolution of all analytes was achieved (Fig. 2). Precision and accuracy for the MS and UV data are given in Table 4, Table 5, respectively. For all standard concentrations at or above the MS LLOQ, the RSD of all compounds using MS data was below 12% (intraday) and 7.0% (interday); and the RE for MS data remained below 7.8% (intraday) and 6.3% (interday) (Table 4). For all standard concentrations at or above the UV LLOQ, the RSD for all compounds using UV data was below 7.2% (intraday) and 8.4% (interday); and the RE for UV data remained below 12% (intraday) and 9.2% (interday) (Table 5). These findings indicate sufficient accuracy and precision for a validated analytical method. According to the FDA Guidance for Industry: Bioanalytical Method Validation, the target values for precision and accuracy of validated methods are $\leq 15\%$ [13]. All measurements in the linear dynamic range for each analyte had a precision and accuracy of $<15\%$ (Table 4, Table 5).

Table 2. Parameters of calibration curves for the MS data.

Analyte	Slope (\pm SD) $\times 10^3$	Intercept (\pm SD) $\times 10^3$	r^2	LLOQ ^a (μ M)	ULOQ ^b (μ M)
1	973.8 (2.9)	11.7 (12)	0.9998	0.0800	10.2
2	315.0 (1.7)	1.4 (7.2)	0.9995	0.0800	10.2
3	1379.5 (4.4)	29.6 (18)	0.9997	0.0800	10.2
4	376.5 (1.4)	-1.1 (6.3)	0.9995	0.160	10.2
5	822.9 (1.9)	22.3 (7.8)	0.9997	0.0800	10.2
6	831.4 (3.5)	25.7 (15)	0.9995	0.0800	10.2
7	798.6 (3.5)	5.9 (15)	0.9996	0.0800	10.2
8	748.5 (2.4)	2.1 (10)	0.9996	0.0800	10.2

^a Lower limit of quantitation.

^b Upper limit of quantitation.

Table 3. Parameters of calibration curves for the UV data.

Analyte	Slope (\pm SD)	Intercept (\pm SD)	r^2	LLOQ ^a (μ M)	ULOQ ^b (μ M)
1	4495.3 (2.6)	15 (260)	1.0000	0.0400	328
2	5068.8 (2.8)	9 (280)	1.0000	0.0400	328
3	5412.3 (3.4)	28 (350)	1.0000	0.0400	328
4	4460.8 (6.3)	-372 (690)	0.9999	0.160	328
5	5474.8 (2.3)	98 (240)	1.0000	0.0400	328
6	5790.5 (2.8)	101 (280)	1.0000	0.0400	328
7	5937.4 (2.9)	67 (290)	1.0000	0.0400	328
8	5156.0 (2.4)	16 (240)	1.0000	0.0400	328

^a Lower limit of quantitation.

^b Upper limit of quantitation.

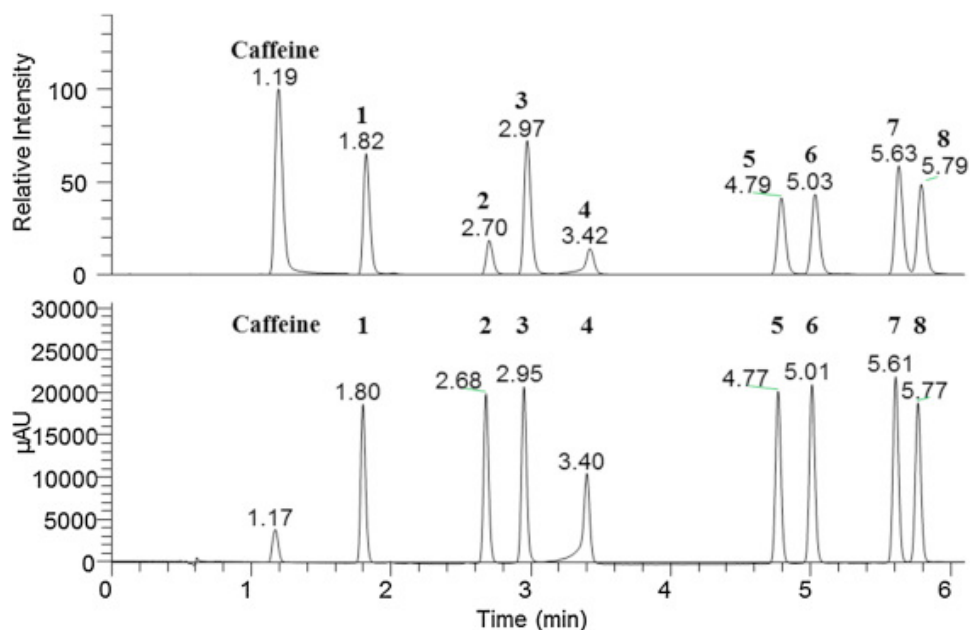


Fig. 2. MS and UV chromatograms of an equimolar mixture of analytes **1-8** at 10.24 μM and the internal standard caffeine^a.

^aPeak at 1.19/1.17 min is caffeine, which was added to monitor for drift in response and retention time.

Table 4. Intraday and interday precision and accuracy using MS detection.

Analyte	Concentration of standard solution injected (μM)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	0.0800	2.0	1.9	2.1	0.51
	0.160	1.2	2.0	1.2	0.65
	0.320	3.0	1.6	2.4	0.82
	0.640	2.8	0.73	0.12	0.63
	1	1.28	0.092	1.3	1.6
	2.56	0.89	0.19	0.37	0.28
	5.12	1.0	0.55	0.18	0.39
	10.2	1.3	0.53	0.34	0.26
	0.0800	7.2	1.6	0.49	2.0
	0.160	8.2	0.76	2.7	0.28
	0.320	2.4	1.5	3.0	1.6
	0.640	1.6	1.4	0.61	2.1
	2	1.28	0.98	2.3	1.1
	2.56	1.1	0.054	2.3	0.93
	5.12	2.5	0.38	1.0	0.074
	10.2	2.5	0.021	0.24	0.25
	0.0800	0.35	7.8	1.6	6.3
	0.160	2.8	0.85	1.5	0.64
	0.320	0.83	0.62	0.99	0.52
	0.640	1.7	3.8	0.95	3.4
	3	1.28	0.60	2.4	0.51
	2.56	0.50	0.47	1.2	0.69
	5.12	1.6	0.79	0.090	0.89
	10.2	0.34	1.0	0.36	1.1

Analyte	Concentration of standard solution injected (μM)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	0.160	4.4	6.5	7.0	1.4
	0.320	3.4	5.6	5.2	0.40
	0.640	5.1	0.25	0.33	0.012
4	1.28	2.6	0.55	1.3	0.50
	2.56	0.45	1.6	1.7	0.37
	5.12	2.7	0.95	0.70	0.45
	10.2	0.64	0.056	0.57	0.37
	0.0800	8.0	1.7	2.4	0.20
	0.160	6.1	7.2	5.7	1.1
	0.320	1.9	4.4	3.8	0.70
	0.640	3.1	3.0	1.5	1.3
5	1.28	1.7	0.019	2.3	0.74
	2.56	0.45	2.0	1.4	0.59
	5.12	1.5	0.28	1.1	0.17
	10.2	0.22	0.41	0.67	0.070
	0.0800	12	0.71	4.5	4.4
	0.160	9.5	0.0050	1.9	1.4
	0.320	4.9	1.0	2.3	3.5
	0.640	4.9	0.79	4.3	1.0
6	1.28	2.9	1.5	0.66	1.0
	2.56	2.4	1.2	0.87	0.30
	5.12	1.5	0.24	0.63	0.33
	10.2	1.9	0.032	0.55	0.079
	0.0800	3.7	5.9	5.9	1.5
	0.160	7.9	4.3	3.0	1.3
	0.320	3.1	3.0	1.7	1.1
	0.640	3.2	1.3	2.0	0.98
7	1.28	0.11	0.12	1.5	0.22
	2.56	1.3	0.92	0.92	0.75
	5.12	2.1	1.7	0.50	1.2
	10.2	1.5	0.59	0.56	0.38
	0.0800	4.5	2.7	0.94	1.9
	0.160	5.4	7.6	5.1	2.4
	0.320	2.8	3.5	1.8	1.7
	0.640	3.6	3.8	3.0	0.72
8	1.28	2.0	0.58	1.9	1.0
	2.56	1.6	2.5	1.1	1.8
	5.12	0.48	0.47	0.23	0.68
	10.2	1.3	0.20	0.41	0.16

Table 5. Intraday and interday precision and accuracy using UV detection.

Analyte	Concentration of standard solution injected (μM)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	0.0400	1.8	2.4	6.0	1.5
	0.0800	6.6	6.4	0.12	6.5
	0.160	1.5	1.4	4.7	0.95

Analyte	Concentration of standard solution injected (μM)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	0.320	1.4	1.2	0.97	0.11
	0.640	0.36	0.33	1.5	1.2
	1.28	0.088	0.40	1.4	2.0
	2.56	0.25	0.72	0.85	1.7
1	5.12	1.2	0.50	0.90	1.5
	10.2	0.22	0.70	0.41	1.2
	20.5	0.52	0.78	0.32	0.94
	41.0	0.037	1.1	0.42	0.70
	81.9	0.033	0.19	0.23	0.26
	164	0.067	0.051	0.27	0.073
	328	0.21	0.29	0.061	0.26
	0.0400	5.8	0.89	3.7	2.8
	0.0800	0.49	0.43	1.3	0.86
	0.160	1.3	6.0	1.6	4.4
	0.320	1.7	0.010	0.19	0.020
	0.640	0.94	0.92	1.0	0.24
	1.28	0.47	1.2	1.2	1.4
	2.56	0.38	1.0	0.85	1.4
2	5.12	1.4	1.5	0.74	1.2
	10.2	0.47	1.1	0.33	1.0
	20.5	0.61	1.0	0.49	0.75
	41.0	0.12	0.88	0.76	0.48
	81.9	0.12	0.066	0.27	0.011
	164	0.038	0.031	0.14	0.042
	328	0.21	0.27	0.16	0.15
	0.0400	2.9	11	2.8	8.5
	0.0800	5.3	3.8	3.5	1.1
	0.160	3.7	3.9	0.80	3.4
	0.320	1.7	0.11	0.75	0.23
	0.640	0.94	1.4	1.7	1.2
	1.28	2.8	1.6	1.2	2.4
	2.56	0.087	2.2	0.55	2.3
3	5.12	1.3	2.1	0.63	1.9
	10.2	0.52	1.4	0.26	1.4
	20.5	1.0	1.5	0.65	1.1
	41.0	0.10	0.90	0.75	0.52
	81.9	0.16	0.019	0.30	0.050
	164	0.063	0.057	0.15	0.053
	328	0.20	0.33	0.19	0.20
	0.160	0.91	12	2.2	9.2
	0.320	1.7	2.5	4.2	4.9
	0.640	1.2	0.58	1.2	2.0
	1.28	4.3	1.4	1.7	2.9
	2.56	0.49	2.5	2.0	3.0
4	5.12	1.0	3.4	0.61	3.4
	10.2	1.3	3.6	0.078	3.6
	20.5	1.1	2.5	0.50	2.2

Analyte	Concentration of standard solution injected (µM)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	41.0	0.49	1.8	0.50	1.4
	81.9	0.0076	0.23	0.092	0.18
	164	0.66	0.15	0.20	0.10
	328	0.20	0.69	0.23	0.59
	0.0400	4.4	5.0	8.4	0.31
	0.0800	2.1	0.82	3.1	1.6
	0.160	1.1	1.4	2.8	1.8
	0.320	2.3	1.3	0.20	1.2
	0.640	1.4	1.2	1.8	0.26
	1.28	0.88	0.29	0.82	0.32
	2.56	0.66	0.69	0.81	0.67
5	5.12	1.1	0.48	0.29	0.60
	10.2	0.29	0.16	0.35	0.0013
	20.5	0.65	0.62	0.34	0.33
	41.0	0.21	0.78	0.85	0.40
	81.9	0.15	0.10	0.35	0.0011
	164	0.10	0.0085	0.17	0.027
	328	0.21	0.18	0.16	0.072
	0.0400	4.4	7.1	6.1	0.97
	0.0800	1.0	4.7	4.0	0.47
	0.160	2.6	4.2	1.2	3.5
	0.320	0.92	2.8	2.3	0.70
	0.640	0.96	0.52	0.99	0.31
	1.28	0.84	0.86	0.82	0.37
	2.56	0.65	1.1	0.81	0.95
6	5.12	0.84	0.59	0.43	0.83
	10.2	0.15	0.13	0.43	0.17
	20.5	0.64	0.89	0.28	0.59
	41.0	0.21	0.93	0.95	0.55
	81.9	0.14	0.12	0.38	0.032
	164	0.11	0.015	0.19	0.056
	328	0.21	0.21	0.17	0.11
	0.0400	5.8	3.5	2.3	1.4
	0.0800	6.8	6.5	2.9	3.4
	0.160	2.9	4.4	3.4	3.2
	0.320	1.9	1.5	1.2	0.29
	0.640	0.75	0.16	1.2	0.40
	1.28	1.2	1.2	1.4	0.38
	2.56	0.76	0.16	1.0	0.078
7	5.12	1.1	0.15	0.38	0.20
	10.2	0.42	0.27	0.31	0.013
	20.5	0.47	0.60	0.35	0.40
	41.0	0.051	0.93	0.88	0.43
	81.9	0.18	0.11	0.39	0.036
	164	0.13	0.037	0.18	0.038
	328	0.26	0.18	0.18	0.070

Analyte	Concentration of standard solution injected (μM)	Intraday		Interday	
		RSD (%)	RE (%)	RSD (%)	RE (%)
	0.0400	7.2	5.8	1.0	4.8
	0.0800	4.1	1.4	6.1	0.62
	0.160	1.8	2.5	1.2	2.4
	0.320	0.035	1.5	1.0	0.28
	0.640	1.0	1.5	1.4	1.2
	1.28	0.55	1.4	1.6	0.18
	2.56	0.93	0.46	0.99	0.39
8	5.12	1.3	0.65	0.48	0.16
	10.2	0.62	0.67	0.32	0.41
	20.5	0.53	0.55	0.35	0.39
	41.0	0.061	0.92	0.91	0.41
	81.9	0.21	0.11	0.41	0.055
	164	0.14	0.057	0.18	0.016
	328	0.25	0.16	0.18	0.059

3.2. Comparison of linear dynamic range with MS and UV detection

One of the goals of this study was to compare MS and UV detectors for quantitative analysis of silymarin components (Fig. 2). The MS and UV detection techniques provided different linear dynamic ranges (Table 2, Table 3). The linear dynamic range for the MS analyses was determined to be 0.0800-10.2 μM for all compounds except compound **4**, which demonstrated a linear dynamic range of 0.160-10.2 μM (Table 2). Above a concentration of 10.2 μM , signal saturation was observed with MS analysis for all of the silymarin constituents investigated. This saturation was not observed up to the highest concentration investigated when the UV detector was employed. For the UV data, the linear dynamic range was 0.0400–328 μM for all compounds except compound **4**, and 0.160–328 μM for compound **4** (Table 3). Saturation at high concentration with MS analysis, particularly of highly polar small molecules, is a common occurrence [14]. However, contrary to the results observed in this study, it is generally the case that MS detection provides lower LLOQ than those observed with UV. Additional ionization methods were evaluated (positive and negative ESI and APCI) and positive mode HESI demonstrated the best sensitivity at the flow rates used in this study. For these studies, the S/N was observed to be higher with the use of the MS detector than the UV detector (Fig. S1). However, peak areas at low concentrations for multiple injections with MS were less repeatable than those measured with UV, leading to a better LLOQ with the UV detector.

3.3. Quantitative analysis of compounds in silymarin mixtures

A series of five commercial silymarin preparations from three different manufacturers was analyzed to determine their total content of compounds **1** through **8** as well as the absolute quantity of each compound individually in the mixtures. The composition of these mixtures varied significantly (at the 95% confidence interval, according to t-test, Table 6), both between different manufacturers and between batches from the same manufacturer. The two batches of silymarin extracts from Madaus showed the highest total content of all eight analytes (62% and 70% by MS), while the two silymarin extracts from Sigma showed the lowest total analyte content (47% and 54%), and the Indena silymarin extract showed a total analyte content of 57%. For compound **4**, the percent composition differed more between Madaus batches (1.4% and

14%) than did the average percent composition for compound **4** for each of the manufacturers (2.5% for Sigma, 7.0% for Indena, and 7.8% for Madaus). These data, which were confirmed with both MS and UV detection (see Section 3.4), reaffirm previous studies of the variability amongst different commercial preparations of silymarin [15], which could be related to differences in growth conditions for the *S. marianum* plants [16] or general inconsistencies in extraction/processing procedures observed with many herbal medicines [17], [18]. For biological studies with *S. marianum* preparations, it is imperative to consider batch- and vendor-specific variation in individual and total flavonolignan content.

Table 6. Comparison of UV and MS results for silymarin extracts. Percent composition of compounds **1–8** in various commercial silymarin extracts.

Extract		Total ^a	1	2	3	4	5	6	7	8
Madaus 37501	UV	67	2.71 (0.01)	1.89 (0.02)	8.63 (0.14)	11.00 (0.03)	13.91 (0.02)	19.49 (0.08)	5.48 (0.02)	3.46 (0.02)
	MS	70	2.65 (0.04)	1.95 (0.05)	8.79 (0.07)	14.18 (0.10)	13.85 (0.15)	19.38 (0.36)	5.58 (0.05)	3.53 (0.07)
Madaus 286061	UV	63	2.84 (0.04)	0.54 (0.01)	13.40 (0.09)	1.43 (0.02)	15.29 (0.05)	22.62 (0.13)	4.56 (0.03)	1.97 (0.02)
	MS	62	2.81 (0.08)	0.52 (0.04)	13.33 (0.09)	1.44 (0.09)	15.17 (0.20)	ALR	4.64 (0.07)	1.94 (0.03)
Indena 27691	UV	56	4.13 (0.02)	1.30 (0.01)	11.53 (0.06)	5.48 (0.04)	10.16 (0.02)	15.94 (0.11)	4.917 (0.001)	2.50 (0.02)
	MS	57	4.13 (0.05)	1.22 (0.05)	11.60 (0.05)	7.02 (0.01)	10.29 (0.11)	15.68 (0.04)	4.92 (0.14)	2.52 (0.01)
Sigma BCBJ0393V	UV	46	3.17 (0.01)	0.84 (0.01)	10.15 (0.05)	3.27 (0.04)	9.17 (0.04)	13.60 (0.04)	3.84 (0.01)	1.97 (0.04)
	MS	47	3.00 (0.08)	0.89 (0.06)	10.25 (0.05)	4.05 (0.07)	9.20 (0.07)	13.93 (0.20)	3.87 (0.07)	1.99 (0.09)
Sigma 05503PG	UV	54	2.60 (0.03)	0.437 (0.003)	13.14 (0.05)	0.83 (0.02)	12.50 (0.03)	18.42 (0.06)	4.20 (0.01)	1.62 (0.01)
	MS	54	2.61 (0.01)	0.40 (0.02)	13.08 (0.05)	0.82 (0.05)	12.51 (0.06)	18.49 (0.24)	4.22 (0.08)	1.70 (0.04)

Shaded values for percent composition indicate a statistically significant difference (at the 95% confidence interval) between the percent composition measured by MS versus UV detection according to the t-test. Unshaded values showed no statistically significant difference between MS and UV data.

ALR = above linear range of the MS detection method.

BQL = below quantitation limit.

ND = not detected.

^a Total percentage of the silymarin extract contributed by the eight compounds being measured in this study.

3.4. Consistency of MS and UV results for quantitative analysis

In most cases, the MS and UV data provided very similar results for the total content of compounds **1–8** in silymarin extracts (Table 6). For all compounds, with the exception of compound **4**, no statistically significant difference was observed between concentrations determined with MS and UV (at the 95% confidence level, according to t-test) (Table 6). Significant differences were observed between MS and UV data for compound **4**, due to matrix interference in the UV data as described in Section 3.5.

3.5. Matrix effects

Matrix effects were not observed in either MS (Fig. S2) or UV (Fig. S3) analysis for all of the compounds (1–8), except for compound 4, which exhibited a clear matrix effect in the UV analysis (Table 7). This matrix effect can be ascribed to contaminants with UV absorbance that co-elute in the peak front for compound 4 (Fig. S3). The UV contaminants are of different mass than compound 4, and are therefore, resolved from this compound with the MS detector and do not interfere with MS analysis (Fig. S2). For this reason, MS detection appears to be preferable for quantitative analysis of compound 4.

Table 7. Examination of matrix effects by UV and MS for silymarin extracts. Each value represents the percent of the spiked analyte that was observed.^a

Extract		1	2	3	4	5	6	7	8
Madaus 37501	UV	101	101	100	80	101	99	100	100
	MS	99	98	93	82	89	86	99	92
Madaus 286061	UV	99	101	100	76	96	94	99	102
	MS	97	99	87	94	87	75	99	95
Indena 27691	UV	103	102	105	80	106	106	103	103
	MS	99	102	91	98	99	97	100	99
Sigma BCBJ0393V	UV	103	102	108	78	105	108	104	104
	MS	102	100	97	97	99	96	104	100
Sigma 05503PG	UV	101	100	103	76	102	104	100	101
	MS	100	98	90	95	94	83	102	94

^a Values close to 100 indicate no matrix effect observed.

Matrix interference due to ionization suppression is a common problem in analyses via mass spectrometry [19]. In the case of silymarin, however, the lack of matrix interference indicates that ionization suppression is not an issue. This is likely due to the fact that the extract is somewhat purified by the manufacturer. Thus, either MS or UV appear to be acceptable detection techniques for quantitative analysis of silymarin components, with the exception of compound 4.

3.6. Stability in DMSO

A series of samples of the pure compounds from silymarin at 500 μ M were examined by UHPLC-UV (after being exposed to DMSO at room temperature for 6 months). These DMSO-exposed sample chromatograms are compared for analytes 1–4 in Fig. S4 and for analytes 5–8 in Fig. S5 with an identical analysis of the freshly prepared samples of the same batches of compounds that had been stored at 4 °C as a dry powder over the same time period. For each compound, a single new peak was observed in the DMSO-exposed sample. For compounds 3, and 5–8, the new degradation peak was greater than 8% of the total area. For the samples stored dry, no degradation products were detected. Consistent with similar findings on other classes of natural products [20], these data suggest that dry storage of silymarin components is preferable to storage in DMSO for maintaining compound integrity. However, for the standard and QC samples used in the quantitative study, no degradation peaks were observed, indicating stability in 1:1CH₃CN:H₂O over the time frame of the quantitative analysis.

4. Conclusion

In summary, herein is described a rigorous, validated method for the quantitative analysis of eight silymarin components in complex extracts. This method should be applicable to other *S. marianum* preparations, provided that appropriate controls are included to evaluate potential interference that may occur in more complex matrices [19]. For the studies described herein, both mass spectrometry and UV detection were effective for quantitative analysis. The UV detection method had the advantage of an expanded linear dynamic range, particularly at high concentrations, whereas the MS method was less prone to matrix interference for compound 4. Additionally, data are provided that indicate the importance of method of storage for maintaining integrity and biological activity of silymarin constituents. Not surprisingly, dry storage of constituents was preferable to prolonged storage in DMSO. Finally, these studies indicated that significant variation is observed in bioactive component concentrations for silymarin extract preparations among different manufacturers and even from different batches from the same manufacturer. Thus, analytical characterization is critical to establish constituent levels in silymarin preparations prior to conducting biological evaluations. The method published herein should prove useful towards conducting this characterization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2016.04.028>.

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