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Determination of Silymarin Flavonoids by HPLC and LC-MS and Investigation of Extraction Rate of Silymarin in *Silybum marianum* Fruits by Boiling Water

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A method has been developed for simultaneous separation and determination of seven important silymarin flavonoids by HPLC and LC-ESI/MS. The separation was completed on an ODS column using 1 % aqueous acetic acid solution and methanol as mobile phase with gradient elution. The methanol is better than acetonitrile as the organic modifier in mobile phase for separation of diastereomers of silybin (silybin A and silybin B) and diastereomers of isosilybin (isosilybin A and isosilybin B) was explained from the viewpoint of molecules interaction. The content of silybin and silymarin in *Silybum marianum* seed, defatted seed meal and standardized extract were determined with satisfactory recovery and precision. Furthermore, the extraction rate of silymarin flavonoids in *Silybum marianum* seed with boiling water was evaluated on the basis of the well-verified quantitative method. It is found that more polar flavonoids such as taxifolin and silychristin have good solution in water and the total extraction rates of seven times were more than 85 %, and the solubility of other silymarin flavonoids is relatively low, but the total extraction rates were all more than 30 % for seven times of extraction. The results of this study are in excellent agreement with those of batch extraction with hot water.

Key Words: Milk thistle, Silymarin flavonoid, Boiling water, Extraction rate, HPLC, LC-MS.

INTRODUCTION

Milk thistle (*Silybum marianum* (L.) Gaertneri) (family: Compositae) is an annual or biennial plant, native to the Mediterranean area, which has now spread to other warm and dry regions in the world. *Silybum marianum* has been known since ancient times and recommended in traditional European and Asian medicine, mainly for treatment of liver disorders[1,2]. Active component of this plant is silymarin that is a light yellow or brown yellow

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incompact powder obtained from milk thistle fruit (seed) after defatted and extracted using organic solvents. Main constituents of the standardized silymarin extract on sale are seven flavonoids including taxifolin (TXF), silychristin (SCN), silydianin (SDN), silybinin A (SBNA), silybinin B (SBNB), isosilybinin A (ISBNA) and isosilybinin B (ISBNB) (Fig. 1). Silymarin has been used to cure liver and gallbladder disorders including hepatitis, cirrhosis and jaundice, protect brain and prevent X-ray. Moreover, recent studies[3-6] indicated that it also reduces biliary cholesterol levels, prevents and treats cancer.

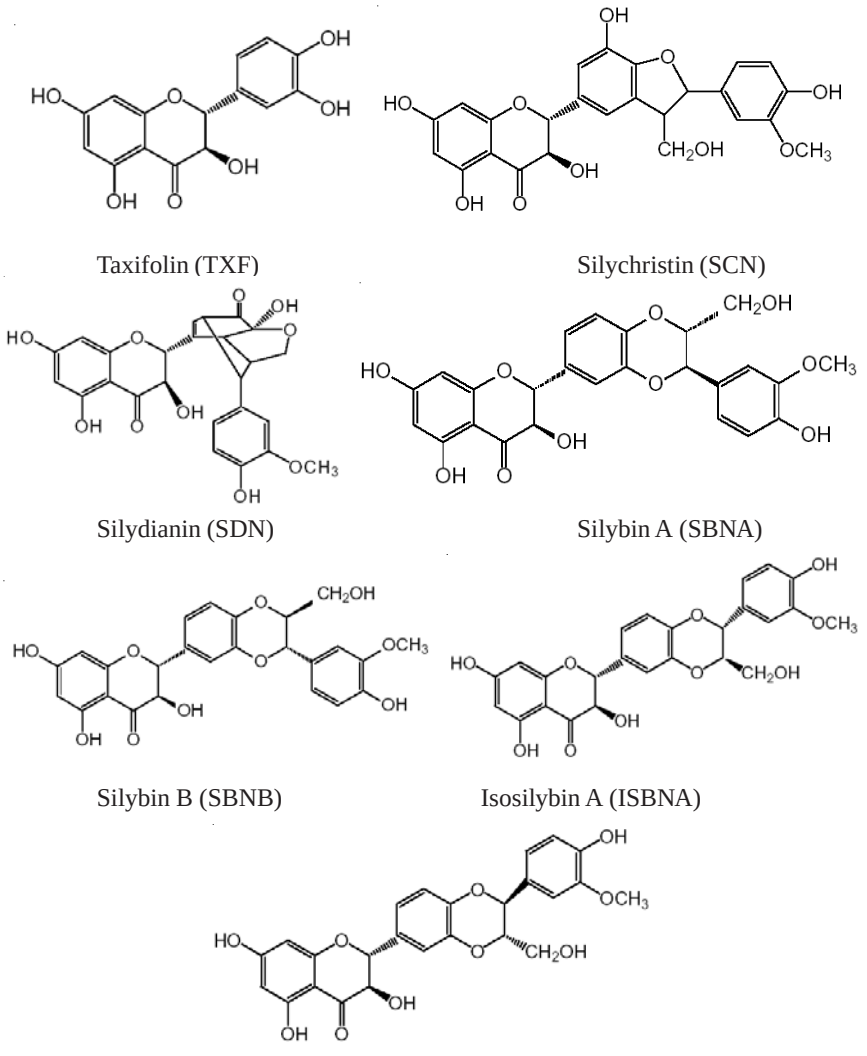

Fig. 1. Chemical structures of seven silymarin flavonoids

Silymarin has been previously analyzed by UV-Vis spectrometry[7], thin layer chromatography (TLC)[8], high performance capillary electrophoresis (HPCE)[9,10] and high performance liquid chromatography (HPLC)[9-18]. The total content of flavonoids rather than that of each flavonoid was determined by UV-Vis because no separation was accomplished. TLC is of limited applicability to this mixture due to poor separation efficiency and tedious process. HPCE is of rapid speed and low sample and solvent consumption, so was employed to analyze silymarin extracts, however, two diastereomers of SBN and ISBN in Quaglia's work[9] and ISBNA and ISBNB in Kvasnicka's work[10] were summation peaks, respectively. HPLC is the most widely utilized method for silymarin flavonoid separation because of high efficiency and excellent reproducibility. Martinelli *et al.*[11] developed an HPLC procedure for measurement of silybin and its conjugates in human plasma and urine. Mascher *et al.*[12] and Rickling *et al.*[13] detected the diastereomers of silybin in human plasma by this technique. Quaglia *et al.*[9] and Kvasnicka *et al.*[10] also used HPLC to separate silymarin flavonoids, however, seven silymarin flavonoids were unable to be completely separated. Six flavonoids (SCN, SDN, SBNA, SBNB, ISBNA and ISBNB) in silymarin were determined by HPLC and confirmed by liquid chromatography-mass spectrometry (LC-MS)[15-17]. Up to now, only Wang *et al.*[14] and Zhao *et al.*[18] applied HPLC to separate seven silymarin flavonoids. However, the resolution in Wang *et al.*'s protocol was unsatisfactory[14] using phosphate buffer solution and methanol as the mobile phase. Zhao *et al.*[18] investigated the HPLC condition and utilized methanol and aqueous formic acid solution as the eluent. Unfortunately, the separation was still not optimized, especially SCN and SDN, SBNA and SBNA, and ISBNA and ISBNB, because no special attention was paid on this seven compounds when eighteen compounds were analyzed in a run. In this paper, a reliable reversed phase HPLC method has been developed for simultaneous determination of silymarin flavonoids based on the good resolution with a gradient elution carefully-chosen.

Traditionally, silymarin was made various preparations after isolated from dry fruits of milk thistle with organic solvents. Clausen's group[19-21] found that hot water is a milder and greener solvent for extracting silymarin flavonoids from milk thistle seeds. In traditional Chinese medicine (TCM) theory, therapeutic efficiency of an herbal medicine is the integrated result of all effective components. Single active component is of much lower efficiency because the co-efficiency disappears. Kvasnicka *et al.*[10] indicated that the higher purity of milk thistle extracts, the lower antioxidant power. In previous work, the present authors investigated the availability of main inorganic elements in *Silybum marianum* fruits in boiling water, which demonstrated that decocting can provide enough trace elements because most of elements of *Silybum marianum* are soluble in boiling water[22]. In this paper, we examined the extraction rates of silymarin flavonoids in *Silybum marianum* seed with boiling water by proposed HPLC method, in

order to further explore the feasibility of extracting effective components from *Silybum marianum* fruit by boiling water.

EXPERIMENTAL

The HPLC system used was Waters Alliance 2695 Separations Module equipped with a vacuum degasser, a quaternary pump, an auto-sampler, and a 996 UV-Vis photodiode-array detector (PDA) (Waters, Milford, MA, USA). The separation was controlled and the chromatograms were recorded by a Waters Empower chromatography manager system. The LC-MSD system consisted of an Agilent 1100 HPLC system, a diode array detector (DAD) and a mass spectrometry detector with electrospray ionization (ESI) source (Agilent, Palo Alto, CA, USA).

Methanol and acetonitrile were of HPLC grade obtained from Jiangsu Hanbang Science and Technology Co. Ltd (Huai'an, Jiangsu, China) and Merck (Rahway, NJ, USA), respectively. Glacial acetic acid, perchloric acid (70-72 %) and petroleum ether (boiling range: 60-90 ºC) were of analytical reagent grade from Nanjing Chemical Reagent Factory (Nanjing, Jiangsu, China). Water was Wahaha purified water (Wahaha Group Ltd., Hangzhou, Zhejiang, China). Standard of SBN (a mixture of SBNA and SBNB, total SBN content ≥ 98 %) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). One standardized silymarin extract (1#) and refined extracts of TXF, SCN, SDN, SBNA, SBNB, ISBNA and ISBNB for retention time reference (the individual purities not certified) were provided by College of Traditional Chinese Pharmacy, China Pharmaceutical University (Nanjing, Jiangsu, China). Another standardized silymarin extract (2#), dried seeds of *Silybum marianum* and defatted seed meal of *Silybum marianum* were provided by Jiushoutang Biological Products Co. Ltd (Taizhou, Jiangsu, China).

Preparation of standard and sample solutions

Solution-A: Standard solutions of SBN: About 10 mg of silybin reference substance was accurately weighed and transferred into a 10 mL volumetric flask. After dissolved with about 8 mL of methanol and sonicated for 20 min, methanol was added to the mark. All other standard solutions of silybin were prepared from this stock solution by diluting with methanol in the further stages of the experiment.

Solution-B: Reference solutions of silymarin flavonoids: About 2.0 mg of individual refined extract of TXF, SCN, SDN, SBNA, SBNB, ISBNA and ISBNB was taken and dissolved in 10 mL methanol, respectively, in order to determine the retention time of each flavonoid.

Solution-C: Solution of standardized extract: About 10 mg of standardized extract was weighed in a 10 mL volumetric flask, then dissolved with methanol to volume after ultrasonication for 20 min.

Solution-D: Methanolic extraction solution of defatted *Silybum marianum* seed meal: 0.5 g of defatted *Silybum marianum* seed meal was extracted with 10 mL methanol for 0.5 h in an ultrasonic bath at 80 ºC. This extracting process was repeated at least four times, until no silymarin flavonoids were detected in last extraction solution, which means the flavonoids are completely isolated from the seeds. Then, all methanolic extraction solutions were combined in a 50 mL volumetric flask and diluted to volume with methanol.

Solution-E: Methanolic extraction solution of *Silybum marianum* seed: This procedure involved a two-step process that is defatting using petroleum ether and extraction using methanol. 0.5 g of powdered *Silybum marianum* seeds was extracted with 5 mL petroleum ether in a soxhlet apparatus for 3 h. This extraction solution was discarded and the residue was extracted again and dried with air. Then the resultant defatted seeds were treated similar to the procedure of solution D.

Boiling water extraction rates experiment: 5.0 g of powdered *Silybum marianum* seeds was mixed with 50 mL boiling water in a 100 mL beaker and then heated in a 100 ºC water bath for 10 min. The supernatant was transferred to a 50 mL volumetric flask. After cooled, add water to the mark. This current infusion was the first decoction. The second to seventh decoctions were made in succession in the same way.

HPLC and LC-MS analysis: HPLC separation and measurement were carried out on a 5 µm Kromasil C_{18} column with the dimension of 150 mm × 4.6 mm (Jiangsu Hanbang) at 30 ºC. A trinary gradient elution employing methanol (A), water (B) and 1 % aqueous acetic acid solution (C) was made at a flow rate of 1.0 mL/min. The linear gradient is: (0 min) 30 % A-50 % B-20 % C, (5 min) 35 % A-45 % B-20 % C, (20 min) 35 % A-45 % B-20 % C, (25 min) 45 % A-35 % B-20 % C, (60 min) 45 % A-35 % B-20 % C. A sample volume of 10 µL was injected throughout the experiment. All solutions were filtered through a cellulose membrane with 0.45 µm aperture prior to injection. The compounds in effluent were monitored at 280 nm. The electrospray ionization source was operated in negative ionization mode (ESI^-). The transmission voltage was set at 70 V. The flow rate and temperature of drying gas was set at 10 L/min and 350 ºC, respectively. Spray gas pressure was 40 psi. Capillary voltage was 4000 V. The full-scan mass spectra were acquired over the range from m/z 105 to m/z 800.

RESULTS AND DISCUSSION

Chromatograms and mass spectra: Fig. 2 gives the typical HPLC chromatograms of silybin reference substance and silymarin samples, in which the peaks numbered from 1 to 7 were well separated. Peaks 1-7

were orderly TXF, SCN, SDN, SBNA, SBNB, ISBNA and ISBNB in comparison with the retention time of individual flavonoid reference. Furthermore, the MS spectra of seven flavonoids were obtained in ESI^- mode (Fig. 3) and the MS data are listed in Table-1. Peak 1 with retention time of 9.3 min shows the quasi-molecular ions $[M-H]^-$ at m/z 303 and $[M-H+Na-H]^-$ at m/z 325 (Fig. 3a). So the peak was identified as TXF. The other six compounds have similar MS spectra so only the MS spectrum of 21.7 min (peak 2) is given (Fig. 3b). The similar quasi-molecular ions indicate the molecular weight of six flavonoids is all 482, that is, the molecular weight of SCN, SDN, SBNA, SBNB, ISBNA and ISBNB. In addition, retention order of peaks 1-7 was in agreement with the reported HPLC profile in literature[14-17].

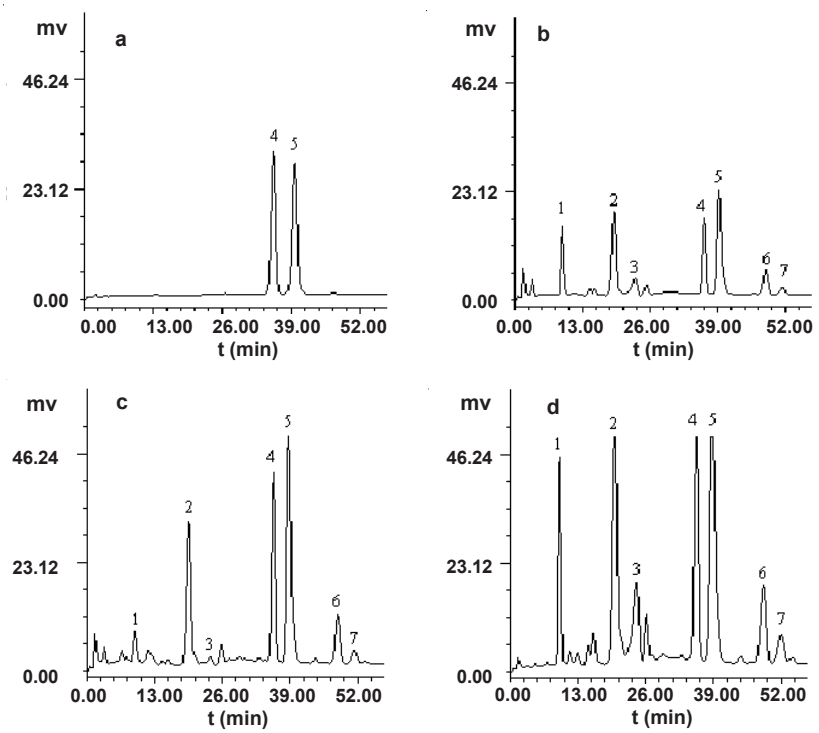

Fig. 2. Chromatograms of silybin standard and silymarin samples. Column: Kromasil C_{18}, 5 µm, 150 mm × 4.6 mm (ID), temperature: 30 ºC. Mobile phase A: methanol; B: water; C: 1 % aqueous acetic acid solution, linear gradient: (0 min) 30 % A-50 % B-20 % C, (5 min) 35 % A-45 % B-20 % C, (20 min) 35 % A-45 % B-20 % C, (25 min) 45 % A-35 % B-20 % C, (60 min) 45 % A-35 % B-20 % C, flow rate: 1.0 mL/min. Injection volume: 10 µL. Wavelength for detection: 280 nm. Panels: a) SBN standard solution; b) methanolic extraction solution of *Silybum marianum* seed; c) methanolic extraction solution of defatted *Silybum marianum* seed meal; d) one of standardized extract solution. Peaks: 1) TXF; 2) SCN; 3) SDN; 4) SBNA; 5) SBNB; 6) ISBNA; 7) ISBNB

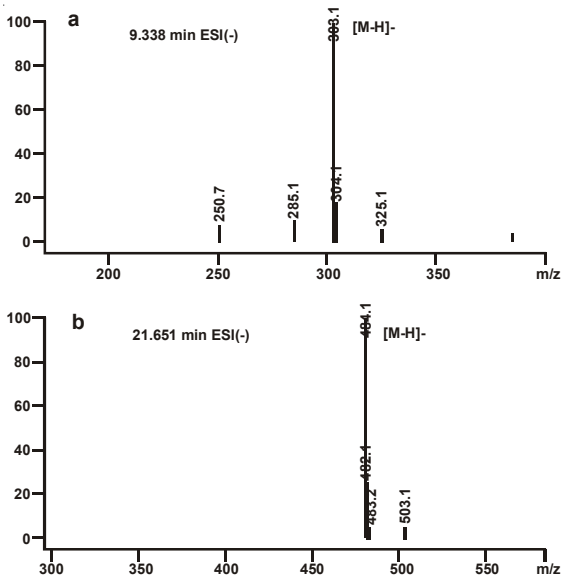

Fig. 3. Mass spectra of selected chromatographic peaks in Fig. 2. Ionization source: ESI^-. Transmission voltage: 70 V. Flow rate of drying gas: 10.0 L/min. Temperature of drying gas: 350 ºC. Spray gas pressure: 40 psi. Capillary voltage: 4000 V. Scanning range: m/z 105 to m/z 800. Panels: a) peak 1 at the retention time of 9.3 min in HPLC chromatogram; b) peak 2 at the retention time of 21.7 min in HPLC chromatogram

TABLE-1
RETENTION TIMES AND MASS/CHARGE RATIOS OF MAIN COMPONENTS IN SILYMARIN SAMPLES

Peak no.	t_R (min)	$[M-H]^-$ (m/z)	$[M-H+Na-H]^-$ (m/z)	m.f.	Name
1	9.3	303	325	$C_{15}H_{12}O_7$	TXF
2	21.7	481	503	$C_{25}H_{22}O_{10}$	SCN
3	25.1	481	503	$C_{25}H_{22}O_{10}$	SDN
4	35.2	481	503	$C_{25}H_{22}O_{10}$	SBNA
5	37.9	481	503	$C_{25}H_{22}O_{10}$	SBNB
6	46.7	481	503	$C_{25}H_{22}O_{10}$	ISBNA
7	49.6	481	503	$C_{25}H_{22}O_{10}$	ISBNB

Validation of HPLC method: Linearity between peak areas from UV-Vis detection at 280 nm *vs.* amounts of SBN was obtained in the concentration range of 1~400 µg/mL. The peak area of SBN was the sum of area of SBNA and SBNB. The linear equations was Area = $31.67 + 2.194 \times 10^4$ C (correlation coefficient = 0.9998). The limit of detection calculated at signal-to-noise ratio of 3 (S/N = 3) was 0.1 µg/mL. Standardized extract solution (solution C), methanolic extraction solution of defatted *Silybum marianum* seed meal (solution D) and methanolic extraction solution of *Silybum*

marianum seed (solution E) were prepared as described. Each sample solution was prepared in triplicate. The content of SBN was quantified by calibration curve method. The total content of silymarin was calculated according to the calibration curve of SBN owing to lack of commercial standards of other flavonoids. Peak area of silymarin was the sum of seven flavonoids area. The precision of intra-day and inter-day (Table-2) were obtained from three analyses, respectively. The recovery was assessed by adding certain amount of standard of silybin to *Silybum marianum* seed and defatted seed meal, respectively and then treated and analyzed in the same procedure. The recovery data were 98.2~101.9 % as indicated in Table-3.

TABLE-2
CONTENTS OF SILYBIN AND SILYMARIN IN SAMPLES (%, n = 3)

Sample	Silybin				Silymarin			
	Intra-day		Inter-day		Intra-day		Inter-day	
	Content	RSD	Content	RSD	Content	RSD	Content	RSD
Seed	0.68	0.12	0.69	0.15	1.37	0.46	1.38	0.50
Meal	1.72	0.76	1.71	0.80	2.80	0.59	2.78	0.60
Extract 1#	25.0	0.19	25.2	0.21	48.2	0.34	48.4	0.36
Extract 2#	21.8	0.32	21.7	0.35	40.8	0.14	40.6	0.16

TABLE-3
RECOVERIES OF SILYBIN IN *Silybum marianum* SEED AND DEFATTED SEED MEAL (n = 3)

Sample	Background	Added (μg/mL)	Found	Recovery	Average (%)	RSD
Seed	19.8	10.0	29.6	98.2	99.2	1.26
	19.8	20.0	39.5	98.8		
	19.8	40.0	60.0	100.6		
Meal	15.8	10.0	25.9	101.2	100.4	1.96
	15.8	20.0	35.4	98.2		
	15.8	30.0	46.4	101.9		

Optimization of chromatographic conditions: In order to choose the optimum chromatographic conditions, various mobile phases were tested. The experimental result displayed that the peak shape of silymarin flavonoids was deformed and the seven flavonoids of interest couldn't be separated with methanol-water and acetonitrile-water as mobile phase whether in isocratic or gradient elution. The peak shape and separation was improved once some acid was added in mobile phase. That's because the hydroxy group of flavonoid partly dissociated in neutral aqueous solution and the dissociation was inhibited when acid was added. All peaks of seven

flavonoids were of good shape and baseline resolved in both methanol-water-1 % aqueous acetic acid solution and methanol-water-0.2 % aqueous perchloric acid solution with gradient elution. However, SCN and SDN, SBNA and SBNB, and ISBNA and ISBNB were not separated when acetonitrile-water-1 % aqueous acetic acid solution as mobile phase in gradient elution. When methanol was added to this eluent, the separation of diastereomers of SBN (SBNA and SBNB), diastereomers of ISBN (ISBNA and ISBNB) could amend, but SCN, SDN and an unidentified peak behind SDN overlapped even with the increase of water percentage in the acetonitrile-containing mobile phase. These experiments presented a fact that methanol was better organic modifier than acetonitrile for separation of silymarin flavonoids, especially for SBNA and SBNB, ISBNA and ISBNB. The separation in most literatures using methanol rather than acetonitrile as organic modifier also proved this conclusion. This is because methanol is a proton solvent, which makes the interaction between methanol and SBNA molecule different from that between methanol and SBNB. Looking at the molecule structure of SBNA and SBNB, SBNA and methanol probably form a stereo ringed structure *via* hydrogen bonds. This crown ether-like macrocyclic structure increases rigidity and stability of the molecule, enlarging the difference between behaviour of SBNA and SBNB in methanol. Consequently, the interaction of SBNA and methanol is much stronger than that between SBNB and methanol. The retention time of SBNA is shorter than SBNB with methanol-water-1 % aqueous acetic acid solution as mobile phase. In addition, the solubility of SBNA in methanol is much higher than SBNB and therefore it yielded the highest SBNA to SBNB ratio when methanol was used as batch extraction solvent[23]. Contrarily, acetonitrile is a non-proton solvent and the interaction to SBNA is the same as that to SBNB, so SBNA and SBNB couldn't be separated only using acetonitrile as organic modifier. In same manner, ISBNA and ISBNB could be separated with methanol other than acetonitrile as modifier (Fig. 4). Methanol-water-1 % aqueous acetic acid solution was chosen for the sake of compatibility with ESI/MS. Furthermore, the separation was best when the content of 1 % aqueous acetic acid solution in mobile phase was 20 %.

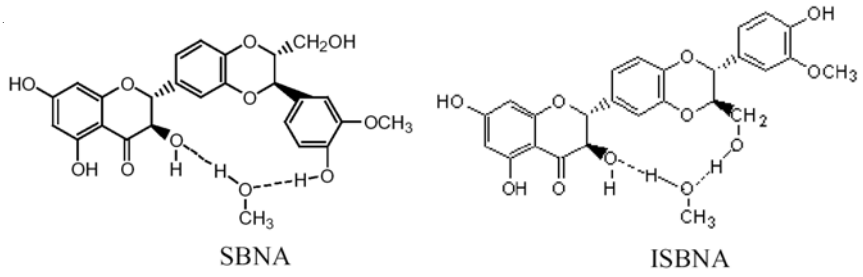

Fig. 4. Possible chemical structures of SBNA and ISBNA in methanol

Extraction rates of silymarin in boiling water: The decoction solutions of *Silybum marianum* seed at different infusion times were analyzed for seven silymarin flavonoids in the same HPLC condition. Obviously, all flavonoids were extracted with boiling water to different extents (Fig. 5). Peak areas of seven flavonoids, as a function of infusion times, were presented in Fig. 6. The extraction rates of flavonoids with boiling water from the first to the seventh time were calculated on the basis of the experiment (Preparation of standard and sample solutions - solution E) that the extraction rates of flavonoids with methanol as extraction solvent are 100 % (Fig. 6 and Table-4).

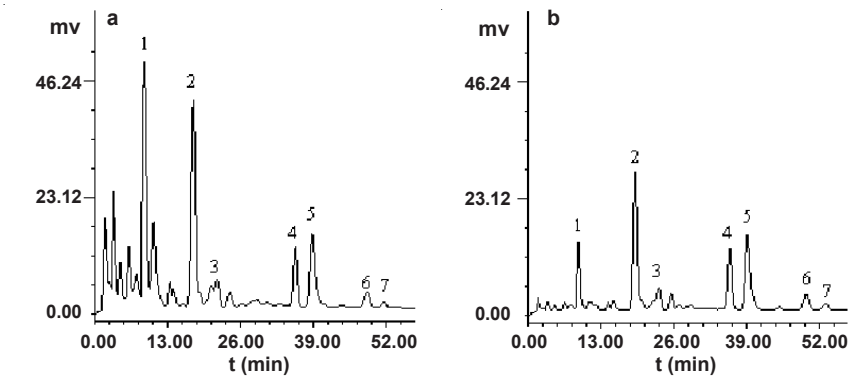

Fig. 5. Chromatograms of tea-like solutions of Silybum marianum seeds at the second time (a) and the fifth time (b). HPLC conditions as in Fig. 2. Peaks: Same as in Fig. 2

Fig. 6 and Table-4 have shown that the extraction rates of all silymarin flavonoids were lowest at the first infusion time except TXF. The extraction rates of SCN and SDN at the second infusion time and decreased gradually with infusion times. The extraction rates of SBNA, SBNB, ISBNA and ISBNB were highest at the fourth time, but dropped down at the sixth and seventh time. The dissolution quantity of TXF and SCN in boiling water was very high, especially TXF, which total extraction rate of seven times was over 100 %. This result indicated that boiling water even is a better extraction solvent than organic solvent for TXF, according with the data of batch extraction[21]. Although the solubility and dissolution quantity of SDN, SBN and ISBN were lower, their total extraction rates of seven times had been more than 30 %, which also agreed with the result of batch extraction[19]. Duan *et al.*[20] reported that the duration for maximal extraction rate would decrease from 200 min to 55 min when the extraction temperature was raised from 100 to 140 ºC, but silymarin flavonoids would decompound. Therefore, extracting with boiling water at 100 ºC is a potential protocol for extraction of effective components in *Silybum marianum*.

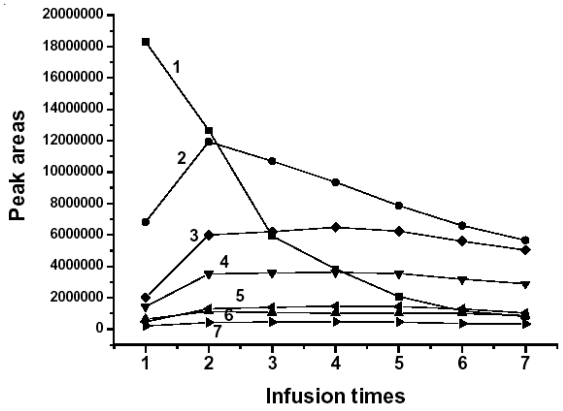

Fig. 6. Relationship between peak areas of silymarin flavonoids in *Silybum marianum* seed and infusion times with boiling water. Lines: 1) TXF; 2) SCN; 3) SBNB; 4) SBNA; 5) ISBNA; 6) SDN; 7) ISBNB

TABLE-4
EXTRACTION RATES OF SEVEN FLAVONOIDS IN *Silybum marianum* SEED AT DIFFERENT INFUSION TIMES (%)

Times	TXF	SCN	SDN	SBNA	SBNB	ISBNA	ISBNB
1	54.6	9.9	3.2	2.6	2.2	1.9	2.2
2	37.8	17.3	5.6	6.4	6.5	5.1	4.8
3	17.7	15.5	5.2	6.5	6.7	5.4	5.0
4	11.4	13.5	5.1	6.6	7.0	5.7	5.4
5	6.2	11.4	5.0	6.4	6.7	5.6	4.9
6	3.5	9.5	5.0	5.8	6.0	5.0	3.9
7	2.4	8.2	4.3	5.2	5.4	4.0	3.7
Total	133.6	85.2	33.4	39.4	40.6	32.7	30.0

Conclusions

A reliable method to separation and analyze seven silymarin flavonoids from *Silybum marianum* has been presented and validated using HPLC with UV combined with ESI/MS detection. This method can be applied not only to the determination of silymarin flavonoids in deferent step of *Silybum marianum* products, but also to the evaluation of extraction efficiency before batch extraction of silymarin flavonoids from *Silybum marianum* seed in boiling water.

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REFERENCES

1. Official Pharmacopoeia Italian, Instituto Poligrafico and Mint Cello Been, Rome, Italy, Vol. 2, edn. 9, p. 1673 (1985).
2. China Pharmaceutical University, Chinese Medicine Dictionary, Chinese Medical Science and Technology Publishing House: Beijing, Vol. 1, p. 963 (1993).
3. W.M. He, J. Yang, G.N. Mao and S.K. Jiang, *Acta Veter. Zootech. Sinica*, **27**, 154 (1996).
4. V. Kren and D. Walterová, *Biomed. Pap.*, **149**, 29 (2005).
5. F. Rainone, *Am. Family Physician*, **72**, 1285 (2005).
6. AHRQ Evidence Reports and Summaries, 21. Milk Thistle: Effects on Liver Disease and Cirrhosis and Clinical Adverse Effects, AHRQ Evidence Reports, Numbers 1-60. http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=hstat1.section.29172 (accessed 23 September 2006).
7. F.Q. Li, J.H. Hu and Q.G. Zhu, *Chin. Traditional Herb Drugs*, **33**, 31 (2002).
8. Q.L. Lin, J.M. Huang and P.S. Xie, *J. Guangdong College of Pharm.*, **14**, 250 (1998).
9. M.G. Quaglia, E. Bossu, E. Donati, G. Mazzanti and A. Brandt, *J. Pharm. Biomed. Anal.*, **19**, 435 (1999).
10. F. Kvasnicka, B. Bíba, R. Ševcík, M. Voldrich and J. Krátká, *J. Chromatogr. A*, **990**, 239 (2003).
11. E.M. Martinelli, P. Morazzoni, S. Livio and E. Ubertin, *J. Liq. Chromatogr.*, **14**, 1285 (1991).
12. H. Mascher, C. Kikuta and R. Weyhenmeyer, *J. Liq. Chromatogr.*, **16**, 2777 (1993).
13. B. Rickling, B. Hans, R. Kramarczyk, G. Krumbiegel and R. Weyhenmeyer, *J. Chromatogr. B*, **670**, 267 (1995).
14. P. Wang, R.Z. Cong, J.D. Wang and L.F. Zhang, *Chin. J. Chromatogr.*, **16**, 510 (1998).
15. T.M. Ding, S.J. Tian, Z.X. Zhang, D.Z. Gu, Y.H. Shi and C.P. Sun, *Chin. J. Pharm. Anal.*, **19**, 304 (1999).
16. T.M. Ding, S.J. Tian, Z.X. Zhang, D.Z. Gu, Y.F. Chen, Y.H. Shi and C.P. Sun, *J. Pharm. Biomed. Anal.*, **26**, 155 (2001).
17. J.I. Lee, B.H. Hsu, D. Wu and J.S. Barrett, *J. Chromatogr. A*, **1116**, 57 (2006).
18. Y. Zhao, B. Chen and S. Yao, *J. Pharm. Biomed. Anal.*, **38**, 564 (2005).
19. J.F. Barreto, S.N. Wallace, D.J. Carrier and E.C. Clausen, *Appl. Biochem. Biotechnol.*, **105-108**, 881 (2003).
20. L. Duan, D.J. Carrier and E.C. Clausen, *Appl. Biochem. Biotechnol.*, **113-116**, 559 (2004).
21. S.N. Wallace, D.J. Carrier and E.C. Clausen, *Phytochem. Anal.*, **16**, 7 (2005).
22. X.L. Cai, Y.J. Chen, L.M. Dai and H.Z. Lian, *Spectros. Spectral Anal.*, **26**, 2125 (2006).
23. S.N. Wallace, D.J. Carrier and E.C. Clausen, *Appl. Biochem. Biotechnol.*, **105-108**, 891 (2003).

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