Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex

Petra Riasová[1,2], Jaroslav Jenčo[3], David Moreno-González[1], Yvan Vander Heyden[2], Debby Mangelings[2], Miroslav Polášek[1], Pavel Jáč[1]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic

[2]Vrije Universiteit Brussel (VUB), Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling, Faculty of Medicine and Pharmacy, Laarbeeklaan 103, 1090 Jette, Brussels, Belgium

[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic

Corresponding author: Dr. Pavel Jáč, Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic

E-mail: jac_p9aa@faf.cuni.cz **Telephone number:** +420 495 067 504

ORCID ID: 0000-0002-8204-1177

Abbreviations: CD – cyclodextrin, EKC – electrokinetic chromatography, 2-HP-β-CD – 2-hydroxypropyl-β-cyclodextrin, IS – internal standard, MeOH – methanol, Ph. Eur. – European Pharmacopoeia, ISB A – isosilybin A, ISB B – isosilybin B, ISCH – isosilychristin, SB – silybin, SB A – silybin A, SB B – silybin B, SCH – silychristin, SCH A – silychristin A, SCH B – silychristin B, SDA – silydianin, TM-β-CD – heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin, TX – taxifolin, USP – United States Pharmacopeia

Keywords: Capillary electrophoresis, Flavonolignans, Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin, Dietary supplements, Silymarin

Abstract

Capillary electrophoresis (CE) method for the baseline separation of structurally similar flavonolignans silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin, and their precursor taxifolin in silymarin complex has been developed and validated. The optimized background electrolyte was 100 mmol L^{-1} boric acid (pH 9.0) containing 5 mmol L^{-1} heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin and 10% (v/v) of methanol. The separation was carried out in an 80.5/72 cm (50 µm id) fused silica capillary at + 25 kV with UV detection at 200 nm. Genistein (10 µg mL^{-1}) was used as internal standard. The resolution between the diastereomers of silybin and isosilybin was 1.73 and 2.59, respectively. The method was validated for each analyte in a concentration range of 2.5-50 µg mL^{-1}. The calibration curves were rectilinear with correlation coefficients ≥ 0.9972. The method was applied to determine flavonolignans in two dietary supplements containing *Silybum marianum* extract. The accuracy was evaluated by comparing the results of the CE analyses of the dietary supplements with those of the reference USP HPLC method. The unpaired t-test did not show a statistically significant difference between the results of both the proposed CE and the reference method ($P > 0.05$, $n = 3$).

1. Introduction

Milk thistle (*Silybum marianum*) is a medicinal plant from the Asteraceae family. It has been traditionally used to treat liver disorders, such as hepatitis, cirrhosis, and liver dysfunction induced by toxins [1–4]. Silymarin extract showed potential anticancer and antioxidant activities and exhibits a positive effect on dyslipidemia [5–8]. Milk thistle extract is obtained from milk thistle fruits or seeds by fat removal and subsequent extraction with suitable solvents [9]. According to Ph.Eur., these solvents can be acetone, ethanol, methanol, and their mixtures with water or pure ethyl acetate [10]. Extract derived from milk thistle (silymarin) is a mixture composed mainly of structurally similar flavonolignans. The total number of flavonolignans found in milk thistle is 23, including at least two pairs of diastereomers [11]. The content of the main flavonolignans, namely silybin A (SB A), silybin B (SB B), isosilybin A (ISB A), isosilybin B (ISB B), silychristin (SCH), and silydianin (SDA) (Fig. 1) is defined in the European Pharmacopoeia (Ph. Eur.) 10th Edition [10], as well as in the United States Pharmacopeia (USP) 39th Edition [9]. Silymarin should consist of 20 - 45% of SCH and SDA, 40 – 65% of SB A and SB B, and 10 – 20% of ISB A and ISB B [9,10]. According to the Ph. Eur., silymarin content in a dried extract of *Silybum marianum* should fall within the range of 30 to 65% [10]. Pharmaceutical preparations with silymarin should contain 90 – 110% of its labeled amount [10]. The same criterion applies to dietary supplements [9].

HPLC has been generally used for the analysis of flavonolignans in *Silybum marianum* [11]. C-18 and other types of reversed-phase stationary phases have been typically applied because of the moderate hydrophobicity of flavonolignans (log P 2.59 [ChemSpider Search and share chemistry, http://www.chemspider.com/Chemical-Structure.1265998.html?rid=a54956b2-5a69-4e9d-9cc8-fb1d5b7df616&page_num=0, 2021 (accessed 3 May 2021)]). Typically, the mobile phase consists of a mixture of methanol (MeOH) and acidified water [9,10,12–20]. For instance, the LC method for the analysis of silymarin defined by the Ph. Eur. [10] uses an end-capped octadecylsilyl silica gel stationary phase with a mixture of phosphoric acid, MeOH, and water as mobile phase under gradient elution. Similar separation conditions are also proposed by the USP method [9]. Fibigr et al.

[21] utilized a core-shell column with a pentafluorophenyl stationary phase for silymarin analysis. An LC method with ESI-MS detection was developed in [14]. The six flavonolignans were separated within 25 min under gradient elution on a C18 column with a mobile phase consisting of (A) 5 mmol L^{-1} ammonium acetate (pH 4.0 adjusted with formic acid) and (B) MeOH/water/formic acid (90:10:0.1, v/v/v) [14]. Significant improvement in the separation efficiency and subsequently the resolution of the isomers was enabled using UHPLC-MS/MS based methods [17,20]. The baseline separation of the main flavonolignans was achieved in less than 10 min [17,20].

Fenclova et al. [22] presented the separation and quantification of 11 silymarin compounds using UHPLC coupled with drift tube ion mobility and quadrupole time-of-flight mass spectrometry. The combination of RP-UHPLC and high-resolution MS with ion mobility enables unique selectivity to separate and quantify less abundant flavonolignans besides the main compounds and interferences. This approach represents the “high-end” method for silymarin fingerprinting.

Capillary electrophoresis (CE) can be considered as an attractive alternative to the chromatographic techniques for the determination of silymarin complex since it provides several advantages over LC, including higher separation efficiencies, lower reagent consumption, smaller sample volumes, and reduced environmental impact [23]. Up till now, two CE methods for the separation of silymarin flavonolignans have been published [24,25]. In the former, the samples were analyzed using an uncoated capillary with a BGE of pH 9.0, containing 100 mmol L^{-1} sodium tetraborate, 100 mmol L^{-1} boric acid, 12 mmol L^{-1} dimethyl-β-cyclodextrin, and 15% MeOH [24]. The separation of both SB and ISB diastereomers was not achieved under these conditions [24]. The latter proposed the use of a fluorinated ethylene-propylene copolymer capillary with carrier electrolyte consisting of an aqueous solution comprising 10 mmol L^{-1} ε-aminocaproic acid, 100 mmol L^{-1} ammonium hydroxide, 0.5% polyvinylpyrrolidone, and 0.1% hydroxyethylcellulose [25]. This CE method enabled the separation of SB A and SB B, while the ISB diastereomers remained unresolved. In summary, these two published CE methods did not allow to achieve baseline separation of all analytes.

It is well known that the micellar electrokinetic chromatography (MEKC) mode offers a different migration behavior due to the different interactions between the analytes and the micelles [26]. Therefore, the full separation of silymarin compounds may possibly be achieved. On the other hand, the addition of cyclodextrins (CD) to the BGE could improve the resolution between peaks because CD can form complexes with molecules based on their inclusion into the hydrophobic cavity [27].

Our work aimed at the development of a CE method for the complete separation of all six main flavonolignans of the silymarin complex and the flavonoid taxifolin (TX). For the separation of SB A/B and ISB A/B diastereomers, we examined different approaches, including conventional capillary zone electrophoresis (CZE), MEKC, CD-MEKC, and electrokinetic chromatography (EKC). We discuss in detail the method development and different factors affecting selectivity. The optimized method was validated in terms of range, linearity, precision, and accuracy, and applied to the assay of two dietary supplements containing silymarin.

2. Materials and methods

2.1 Chemicals

The standards of silybin (SB, 92.53%), SB B (≥95%), ISB A (≥95%), ISB B (≥90%), SCH (≥95%), SDA (≥95%), TX (≥85%), and genistein (≥98%), used as the internal standard (IS) for the validation, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of SB B, ISB A, ISB B, SCH, and SDA, were also supplied by PhytoLab (Vestenbergsgreuth, Germany).

Dietary supplements: Preparation 1 (150 mg of milk thistle extract/tablet) and Preparation 2 (175 mg of milk thistle extract/140 mg of silymarin/tablet) were purchased in a local pharmacy.

All aqueous solutions were prepared in 18 MΩ.cm^{-1} water generated by a Merck Millipore MilliQ water reverse osmosis system (Millipore, Bedford, MA, USA). Chemicals used for the preparation of BGE were purchased from Sigma-Aldrich. These were boric acid (≥99.5%), sodium dodecyl sulfate (≥98.5%), α-CD, β-CD, γ-CD, 2-hydroxypropyl-γ-CD, 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD), and

heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD). Heptakis(2,6-di-*O*-methyl)-β-CD was obtained from Fluka Chemie (Germany). MeOH (HPLC, gradient grade) was supplied by VWR International (Stříbrná Skalice, Czech Republic). Ortho-phosphoric acid (86%, p.a.), used for HPLC analysis, was purchased from PENTA (Chrudim, Czech Republic).

2.2 Instrumentation

All CE experiments were carried out using an Agilent CE 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector. ChemStation software (version B.04.03-SP1) was used for data acquisition. A Sentron SI400 pH meter with MicroFET pH probe (Sentron Europe B.V., Leek, The Netherlands) was used for pH measurements. Samples were processed by using a MIKRO 220 R centrifuge (Hettich, Tuttlingen, Germany) and a Promax 1020 platform shaker (Heidolph Instruments, Schwabach, Germany).

2.3 Preparation of the optimal BGE

The BGE was prepared by dissolving the appropriate amount of boric acid (309.2 mg) in approx. 25 mL of ultra-pure water and its pH was adjusted to 9.0 with 1 mol L^{-1} NaOH. This solution was completely transferred to a 50.0 mL volumetric flask, 5.0 mL of MeOH was added, and the volume was made up to the 50 mL mark with ultra-pure water to obtain a solution with 100 mmol L^{-1} of boric acid. A weighed amount of TM-β-CD (71.5 mg), corresponding to its final concentration of 5 mmol L^{-1}, was dissolved in 10.0 mL of the preliminarily prepared electrolyte.

The BGEs used during the method development were prepared similarly by weighing the appropriate amounts of reagents.

2.4 Preparation of stock and sample solutions

The stock solutions of flavonolignan standards were prepared by dissolving approximately 1 mg of the standard in 1.0 mL MeOH. The stock solution of the internal standard was prepared accordingly. The working and calibration solutions were prepared by the diluting stock solutions with 25% (v/v)

aqueous MeOH for the optimization and with 100% (v/v) MeOH for the validation to achieve the required final concentrations of flavonolignans and internal standard.

The dietary supplement solutions for CE analysis were prepared as follows: Approximately 5 mg homogenized tablet powder was extracted with 8.0 mL MeOH in two steps. Initially, 3.92 mL MeOH fortified with 80 µL IS stock solution (concentration 1 mg mL^{-1}) was placed in a centrifuge tube with the powdered tablets. The mixture was agitated on a mechanical shaker for 10 min and centrifuged for 5 min at 6,000 rpm (3743 x g). Then 3.95 mL of the extract was transferred into an empty centrifuge tube. In the next step, 4.0. mL pure MeOH was added to the tablet powder to carry out the second extraction. The extract from the first step was added to the second extract, which still contained tablet powder (total volume was 8.0 mL, the final concentration of IS was 10 µg mL^{-1}), centrifuged at 6,000 rpm (3743 x g), and analyzed using the developed CE method.

2.5 CE separation conditions

The separation was carried out in a fused silica capillary (Polymicro Technologies - Molex, Phoenix, USA) with 50 µm id, 363 µm od, and a total length of 80.5 cm (effective length 72 cm). The samples were hydrodynamically injected by applying a pressure of 5000 Pa (50 mbar) for 6 s. The capillary temperature was maintained at 25 °C, and the applied voltage was + 25 kV. The detection wavelength was 200 nm.

A new capillary was conditioned by rinsing with 1 mol L^{-1} NaOH (30 min), 0.1 mol L^{-1} NaOH (30 min), and ultra-pure water (10 min) before the first use. Daily, the capillary was flushed with MeOH (5 min), 1 mol L^{-1} NaOH (5 min), 0.1 mol L^{-1} NaOH (5 min), and ultra-pure water (5 min) before the first analysis and with MeOH (10 min), 0.1 mol L^{-1} NaOH (10 min), and ultra-pure water (10 min) after the last one. Before each measurement, the capillary was rinsed with 0.1 mol L^{-1} NaOH (2 min), ultra-pure water (2 min), and BGE (2 min). Between the rinsing of the capillary with BGE and the sample injection, the inlet electrode was washed eight times with MeOH to prevent carry-over, sample

deterioration, and contamination. MeOH was placed in four vials, i.e., two washing cycles were carried out in one washing vial.

The optimized BGE was 100 mmol L^{-1} borate buffer at pH 9.0 (i.e., 100 mmol L^{-1} boric acid, pH was adjusted with 1.0 mol L^{-1} NaOH) containing 5 mmol L^{-1} TM-β-CD and 10% (v/v) MeOH. The electrolyte was filtered through a 0.45 µm nylon syringe filter (Agilent Technologies, USA) and degassed in an ultrasonic bath for 10 min.

2.6 Validation

The method was validated in terms of linearity, repeatability, precision, and accuracy. The analytes were identified by comparing their migration times with those of standards and by the standard addition method. For all calculations, the peak areas were corrected by migration time. Quantification of the samples was accomplished by using the internal standard (IS) method, i.e., calculating the ratio corrected area of an analyte / corrected area of internal standard.

Linearity was examined by the analysis of eight calibration solutions covering the concentration range from 2.5 to 50 µg mL^{-1} (2.5, 5, 7.5, 10, 20, 30, 40, and 50 µg mL^{-1}) for all analytes; the calibration solutions were prepared once, each solution was injected in triplicate.

The injection repeatability of migration times and corrected peak area ratios of the analytes and the IS was characterized by the relative standard deviation (RSD (%)) calculated from ten consecutive injections at concentration levels of 5, 7.5, and 10 µg mL^{-1}.

Intermediate precision of the method was expressed as RSD of migration times and corrected peak area ratios calculated from three injections of repeatedly prepared standard solutions (concentration 10 µg mL^{-1}) on three successive days (n = 3).

The accuracy of the method was evaluated by comparing the CE analysis results of Preparations 1 and 2 with the results of the USP HPLC method (see Section 2.7). Three independent assays (each based on triplicate injections) were carried out using both CE and the reference HPLC methods. The

comparison of means was made by the unpaired t-test ($P > 0.05$, n=3) in GraphPad Prism software version 9.0.2. (San Diego, CA, USA).

2.7 Comparative HPLC analysis

2.7.1 Instrumentation

The HPLC analysis was performed using an Agilent 1260 liquid chromatography system consisting of G1311C quaternary pump, G1329B autosampler, G1316A column thermostat compartment, G1365D multiple wavelength detector, and G1364C fraction collector (Agilent Technologies, Santa Clara, California, USA). The OpenLab CDS ChemStation software (version A.02.19, revision C.01.09) (Agilent Technologies) was used for data collection and processing.

2.7.2 Sample preparation

Approximately 1 mg homogenized powdered tablets was extracted with 900 µL MeOH. The mixture was sonicated for 20 min with occasional shaking, cooled to 20°C, and diluted to the final volume of 1000 µL [9].

2.7.3 HPLC separation conditions

The LC method applied USP recommendations [9]. The separation was carried out using a fully porous YMC Triart C18 column (150 × 4.6 mm, 5 µm, 120 Å, L1) supplied by YMC CO., Ltd. (Kyoto, Japan). The mobile phase comprised (A) MeOH, phosphoric acid, H_2O (20:0.5:80, v/v/v), and (B) MeOH, phosphoric acid, H_2O (80:0.5:20, v/v/v) and was delivered using a flow rate of 1 mL min^{-1}. The gradient elution program was as follows: 0 - 5 min: 15% B, 5 -20 min: 15 - 45% B, 20 - 40 min: 45% B, 40 - 41 min: 45 - 15% B, 41 - 55 min: 15% B. The column temperature was held at 40 °C, and the injection volume was 10 µL. The chromatograms were acquired at 288 nm at a data sampling rate of 20 Hz.

2.7.4 Linearity

The linearity of the HPLC method was determined by generating calibration curves for each analyte. The calibration solutions contained 4.0 to 50 μg mL^{-1} of an analyte at seven concentration levels (4, 10, 15, 20, 30, 40, and 50 μg mL^{-1}). Each calibration solution was injected in triplicate.

3. Results and Discussion

3.1 Method development and optimization

3.1.1 Cyclodextrin-modified micellar electrokinetic chromatography

Initially, the separation was optimized in terms of SDS concentration, type and concentration of CD, concentration and pH of the buffer, and the content of MeOH as an organic modifier. The experiments were performed in a fused silica capillary with 50 μm id, total length 48.5 cm, and effective length 40 cm. CZE separation of silybin diastereomers, using borate buffer at pH 9.0 with 10% (v/v) MeOH as organic modifier was evaluated. Initial pH, MeOH content, and detection wavelength (200 nm) were adopted from Quaglia et al. [24]. Three different concentrations of boric acid (25, 50, and 100 mmol L^{-1}) were tested. Nevertheless, silybin diastereomers were not resolved. Both SB A and SB B comigrated as anions in a single zone in alkaline borate buffer. Only 4 peaks were monitored when using the pure borate-based BGE for the separation of all seven analytes. The same observation was also made previously by Quaglia et al. [24]. BGE containing 100 mmol L^{-1} boric acid was chosen for further optimization as a compromise between a short analysis time and an acceptable separation current.

The first stage of the method development focused on the separation of SB diastereomers. Based on a literature survey, the MEKC separation mode with sodium dodecyl sulfate as pseudostationary phase was tested because it was possible to achieve SB separation by means of reversed-phase HPLC. Concentrations of 70, 140, and 220 mmol L^{-1} SDS had no effect on the separation of ISB A and B (Rs = 0). However, an acceptable resolution between SB A and SB B (1.26) was achieved after the addition of 140 mmol L^{-1} SDS to the BGE, without an excessive increase in the separation current (81 μA).

Further increase in SDS concentration resulted in even better resolution (2.00) between SB A and SB B. However, the addition of more surfactant induced a large increase in the electrophoretic current (Table S1), so it was decided to stay with 140 mmol L^{-1} SDS as the final concentration.

In the second stage, the effect of several CD, available in the laboratory, including α-CD, β-CD, γ-CD, 2,6-di-*O*-methyl-β-CD, and 2-HP-β-CD on the separation of the ISB diastereomers was examined without the addition of SDS. The initially tested concentrations of the CD in the BGE were 1.5 and 10 mmol L^{-1}. Partial resolution of ISB A and ISB B was achieved with 1.5 mmol L^{-1} β-CD and 2-HP-β-CD. Thus, their effect on the separation of ISB was examined further at concentration levels between 0.5 and 15 mmol L^{-1} (Table S2). The best resolution between ISB A and ISB B was achieved with 1 mmol L^{-1} 2-HP-β-CD. However, the addition of CD had no influence on the separation of the SB diastereomers.

In the next optimization step, the BGE containing CD was enriched with 140 mmol L^{-1} SDS to investigate the selectivity towards all 4 diastereomers in CD-MEKC separation mode. The combination of SDS and CD in one BGE created competitive interactions of these two pseudostationary phases with the analytes, leading to a negative effect on the resolution. Consequently, the next step was the optimization of the 2-HP-β-CD concentration in the range 1.5 to 10 mmol L^{-1} in the BGE containing 140 mmol L^{-1} SDS, 100 mmol L^{-1} boric acid, pH 9.0, and 10% (v/v) MeOH (Table S3). The best resolution between SB A/SB B and ISB A/ISB B was achieved with the BGE containing 5 mmol L^{-1} 2-HP-β-CD, 140 mmol L^{-1} SDS, 100 mmol L^{-1} boric acid, pH 9.0, and 10% (v/v) MeOH.

After finding separation conditions for the separation of the two diastereomer pairs, the BGE was optimized for all 7 compounds. The effect of the MeOH content was examined in a range of 5 – 15% (v/v) (Table S4). With the best BGE containing 5 mmol L^{-1} 2-HP-β-CD, 140 mmol L^{-1} SDS, 100 mmol L^{-1} boric acid, pH 9.0 and 10% (v/v) MeOH, the resolution between SB A and SB B, and between ISB A and ISB B was 1.33 and 1.62, respectively (Fig. 2 (A)). Significant variations in migration times of SCH

and SDA ranging from 4.80 to 7.09 min and from 5.28 to 8.56 min, respectively, were observed. This led to the comigration of SDA with ISB B and to changes in the migration order of SCH and SDA. Therefore, the CD-MEKC method was not considered for further validation.

3.1.2 Electrokinetic chromatography (EKC)

The optimization of the CE method started again with the addition of TM-β-CD to the alkaline buffer. Its influence on the separation of the flavonolignans was not tested so far. The effect of the TM-β-CD concentration was studied from 1.0 to 15.0 mmol L^{-1}, with 0.5 mmol L^{-1} increments between 5.0 and 7.5 mmol L^{-1} because of the similar resolution attained within this concentration range (Table S5). The TM-β-CD proved to have a quite unique selectivity towards both diastereomers. Satisfactory separation ($Rs \geq 1.40$) of the critical diastereomer pairs was observed at 5 mmol L^{-1} TM-β-CD. To improve the resolution between the SB A/B pair, an additional increase in the capillary length (56 cm effective length and total length 64.5 cm) was necessary. We also examined the effect of pH and MeOH content (Table S6) on the selectivity of the separation. The pH of the buffer varied in the range from 8 to 10, and the influence of 0 to 20% (v/v) MeOH content was studied. Finally, the optimized BGE consisted of 100 mmol L^{-1} borate buffer at pH 9.0 containing 5 mmol L^{-1} TM-β-CD and 10% MeOH, being a compromise between adequate analysis time and good separation of all the flavonolignans.

The final method was transferred to longer capillaries with 72 cm effective length and a total length of 80.5 cm in order to attain baseline separation of all analytes (Table S6). The standard solutions were prepared in 100% MeOH to make them similar to the extraction solvent (pure MeOH). The resolution between the critical diastereomers pairs was ≥ 1.70 under these conditions, indicating baseline resolution (Fig. 2 (B)).

We were able to achieve baseline separation of all 7 analytes with completely different selectivity compared to the work of Quaglia et al. [24], who recorded only 5 peaks for 7 compounds. Similarly, Kvasnička et al. achieved only partial separation of the SB diastereomers resulting in 5 recorded

peaks for 6 compounds (TX was not analyzed)[25]. Hence, the CE baseline separation of the 6 main flavonolignans and taxifolin is demonstrated in this communication for the first time. Moreover, different selectivity towards the individual diastereomers under various CE conditions was examined and discussed: (i) SB diastereomers were separated in the MEKC system, while (ii) ISB diastereomers were resolved using β-CD and HP-β-CD, and (iii) the separation of all 4 diastereomers was achieved using TM-β-CD. These observations provided a valuable insight into the separation capabilities of the tested separation systems.

3.2 Validation of the method

3.2.1. Linearity and range

Good linearity with correlation coefficients $r \geq 0.9972$ was achieved in the examined concentration range 2.5 - 50 μg mL^{-1} for all flavonolignans and taxifolin (Table 1). The LOQ values (S/N = 10) were between 1.0 and 2.1 μg mL^{-1} for all analytes.

3.2.2. Repeatability and precision

The injection repeatability of the method was evaluated as RSD (%) of corrected peak area ratios of an analyte and the IS, and of the migration times of analytes for ten consecutive injections of standard solutions at three concentration levels, i.e., 5, 7.5, and 10 μg mL^{-1}. The RSDs of corrected peak area ratios were ≤ 1.80%, ≤ 1.34%, and ≤ 1.15% at concentrations 5, 7.5, and 10 μg mL^{-1}, respectively. The RSDs of migration times were ≤ 0.92%, ≤ 0.36% , and ≤ 0.25% at concentrations 5, 7.5, and 10 μg mL^{-1} (see Table 1). These results were considered satisfactory.

The intermediate precision of migration times and corrected peak area ratios was evaluated as RSD obtained from three injections of standard solutions prepared on three consecutive days at 10 μg mL^{-1} (n = 3). The RSDs of the migration times varied between 0.97 – 1.60% and of the corrected peak area ratios ranged between 1.31 – 3.28% (see Table 1). These results can be considered acceptable.

3.3 Analysis of dietary supplements and method accuracy

Our method was applied to the analysis of dietary supplements with different declared concentrations of dried *Silybum marianum* extract, available on the Czech market (Table S7). The three independently prepared sample extracts and three calibration standards (2.5, 10, 30 µg mL^{-1}) were injected in triplicate. The content of the analytes in the dietary supplements was quantified by the two-standard calibration technique [28]:

$$x_{spl} = \frac{x_2(y_{spl} - y_1) - x_1(y_{spl} - y_2)}{y_2 - y_1}$$

where x_{spl} is the concentration of the analyte in the sample, x_1 and x_2 are the concentrations of calibration standards, y_1 and y_2 are the average corrected peak area ratios of the analyte and the IS in the calibration standards, and y_{spl} is the average corrected peak ratio of the analyte and the IS in the sample. The calculation for each analyte was done with respect to its response, i.e., y_{spl} is bracketed between y_1 and y_2 (either between 2.5 and 10 µg mL^{-1} or between 10 and 30 µg mL^{-1}). The concentration ranges of the standards were selected to conform with the content of the analyte in the sample.

The precision of the entire method, including the extraction process, was characterized by RSD ≤ 6.5% of the analytes content (calculated from 3 consecutive extractions; n=3). Preparation 1 (Fig. 3 (A)) and Preparation 2 (Fig. 3 (B)) are dietary supplements with 150 mg and 175 mg of dried milk thistle extract, respectively. Additionally, the amount of silymarin declared by the manufacturer in Preparation 2 is 140 mg what corresponds to 80% potency. The amount of silymarin found was 42.8% ± 1.9% and 48.4% ± 1.8%, respectively, which is in accordance with the requirements of Ph. Eur. (30 - 65%) for dried silymarin extracts (Table 2). Moreover, the content of individual flavonolignans, expressed as % of the total silymarin found, agreed with the Ph. Eur and the USP specifications for both preparations (Table S8). In Preparation 2, the content of silymarin found was 60.5% of the labeled silymarin content (i.e., 140 mg). Thus, it is outside of the range required by Ph. Eur. and the USP (90 – 110%). A possible explanation of this finding may be incorrect information on

the label because 80% potency of milk thistle extract is unusually high. Moreover, this preparation fulfills the other criteria on the flavonolignan content set by the pharmacopeias (see Table 2 and S8).

The accuracy of the novel CE method was evaluated by comparison of the results with the HPLC method based on the USP (Section 2.7) [9]. The calibration curves (Table S9) were used for the HPLC quantification of the analytes in the same dietary supplements. The average content of silymarin found by the USP HPLC method in the dried milk thistle extract of Preparation 1 and 2 corresponds to 45.8% ± 4.3% and 50.2% ± 6.3% of the labeled amount, respectively (Table 2). In the case of Preparation 2, the found silymarin content corresponds to 62.8% of the declared amount, which is in reasonable agreement with the CE assay. The unpaired t-test was used to compare the CE and HPLC assays. The results of the test were interpreted by P values that were 0.33 and 0.65 for Preparation 1 and Preparation 2, respectively, indicating no significant difference between the means obtained by the two methods ($P > 0.05$, $n = 3$). The Shapiro-Wilk test for normality of residuals was successful with P values 0.96 and 0.72 for Preparation 1 and Preparation 2, respectively.

The peak purity was checked for all flavonolignan peaks in the electropherograms of Preparation 2 to determine the method selectivity. The values of peak purity calculated by ChemStation were always above the threshold (see Table S 10), i.e., the peaks can be regarded as pure based on the UV-VIS spectrum. In some cases, not enough data points were available to assess the peak purity, especially for SDA. Thus, it was not possible to assess the selectivity for this peak. Similar observations were also made for Preparation 1 (data not shown).

4. Concluding remarks

In this work, we addressed the challenging optimization of a CE method for the complete separation of six main flavonolignans and the flavonoid taxifolin in the silymarin complex for the first time.

Baseline separation of all main compounds was attained with TM-β-CD as BGE additive. Even though the CE separation takes almost 25 min (nearly 34 min including the preconditioning steps), it is still

faster than the USP HPLC (Section 2.7)[9] or the Ph. Eur. methods [10]. The analysis time of the pharmacopeial methods exceeds 50 min without achieving baseline separation of all 6 flavonolignans. A comparison of our CE method with other reported separation alternatives is presented in Table S11. In terms of sensitivity, the proposed method performed similarly to the method of AbouZid et al. [19] but is less sensitive than other LC-UV methods listed in Table S11 [18,21]. The two reported CE methods covered a wider concentration range and achieved better precision. Unlike these two methods, our method allows baseline resolution of all analytes. However, method sensitivity lags compared to UHPLC coupled to a very sensitive detector, such as QTOF [22].

Our CE method can be considered more environmentally friendly compared to LC methods. It fulfills two principles of green analytical chemistry (GAC) since the waste generation is very low (GAC principle No. 7), and CE is a miniaturized technique (GAC principle No. 5) [29]. Finally, the developed EKC-UV method was applied to the determination of the content of silymarin and individual flavonolignans in dietary supplements. We determined the potency of the extract in two supplements and achieved results comparable with the HPLC-based USP method. The results confirmed that the two tested supplements met the requirements of USP and Ph. Eur for milk thistle dried extract [9,10].

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Conflict of Interest

The authors have declared no conflict of interest.

Data Sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE CAPTIONS

Fig. 1 Structures of flavonolignans and taxifolin.

Fig. 2 Electropherograms of flavonolignans under (A) CD-MEKC and (B) EKC separation conditions. CD-MEKC: 5 mmol L^{-1} 2-HP-β-CD, 140 mmol L^{-1} SDS, 100 mmol L^{-1} borate, pH 9.0, and 10% MeOH, fused silica capillary 50 µm id, 48.5/40 cm long. EKC: 5 mmol L^{-1} TM-β-CD, 100 mmol L^{-1} borate, pH 9.0, and 10% MeOH, fused silica capillary 50 µm id, 80.5/72 cm long. Applied voltage: + 25 kV. Detection: 200 nm. The concentration of standards, including IS, was 10 µg mL^{-1}. Peak assignment: 1-SB A, 2-SB B, 3-ISB A, 4-ISB B, 5-SCH, 6-SDA, 7-TX, IS-genistein.

Fig. 3 Electropherogram of (A) Preparation 1 and (B) Preparation 2 extract analyzed under optimal separation conditions (5 mmol L^{-1} TM-β-CD, 100 mmol L^{-1} borate, pH 9.0 and 10% MeOH, fused silica capillary (50 µm id, 80.5/72 cm long), + 25 kV. The UV detection was at 200 nm. Peak assignment: 1-SB A, 2-SB B, 3-ISB A, 4-ISB B, 5-SCH, 6-SDA, 7-TX, IS- Genistein.

Table 1: Validation parameters for individual compounds (concentration range 2.5-50 µg mL^{-1}).

Parameter	Silybin B	Silybin A	Isosilybin B	Isosilybin A	Silychristin	Silydianin	Taxifolin
Slope	0.1185 ± 0.0024	0.1238 ± 0.0024	0.1256 ± 0.0025	0.1393 ± 0.0029	0.1069 ± 0.0020	0.0576 ± 0.0018	0.1118 ± 0.0022
Intercept	0.0465 ± 0.0645	0.0143 ± 0.0634	-0.0469 ± 0.0651	-0.0405 ± 0.0771	-0.0167 ± 0.0521	-0.025 ± 0.0465	-0.0272 ± 0.0594
Correlation coefficient	0.9987	0.9989	0.9988	0.9987	0.9990	0.9972	0.9988
LOQ (µg mL^{-1})	1.3	1.1	1.2	1.0	1.6	2.1	1.7
Repeatability of migration times, RSD (%) (n = 10)	0.05 – 0.70	0.06 – 0.71	0.05 – 0.72	0.06 – 0.73	0.25 – 0.92	0.07 – 0.70	0.08 – 0.73
Repeatability of corrected areas ratio, RSD (%) (n = 10)	0.88 – 0.99	0.65 – 1.15	0.87 - 1.09	0.65 – 1.03	0.85 – 0.93	0.77 – 1.10	0.94 – 1.80
Intermediate precision of migration times, RSD (%) (n = 3)	0.98	0.98	0.97	0.97	1.06	1.41	1.60
Intermediate precision of corrected areas ratio, RSD (%), (n = 3)	1.59	2.09	1.57	1.31	3.28	1.70	2.33

Table 2: Comparison of declared and found content of silymarin in dietary supplements determined by the CE and HPLC methods.

				CE		HPLC	
Food Supplement	**Declared content (mg)**	**Required[a] amount (%)**	**Required[a] amount (mg)**	**Found content (mg)**	**Found content (%)**	**Found content (mg)**	**Found content (%)**
Preparation 1	150 [b]	30-65	45-97.5	64.2 ± 2.9	42.8 ± 1.9	68.8 ± 6.5	45.8 ± 4.3
Preparation 2	175[b]	30-65	52.5-113.8	84.7 ± 3.1	48.4 ± 1.8	87.9 ± 11.0	50.2 ± 6.3
	140[c]	90-110	126-154	84.7 ± 3.1	60.5 ± 2.2	87.9 ± 11.0	62.8 ± 7.8

[a] Ph. Eur.[10]; [b] dried *Silybum marianum* extract; [c] Silymarin

Figure 1

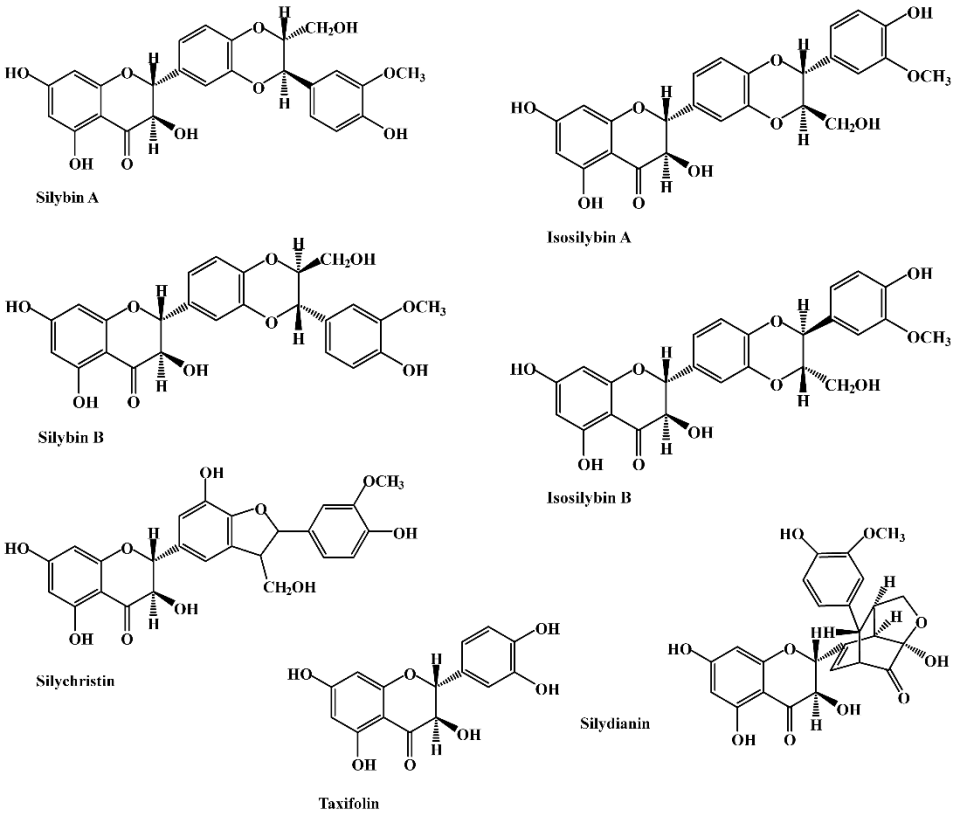
Silybin A
Isosilybin A
Silybin B
Isosilybin B
Silychristin
Taxifolin
Silydianin

Figure 2

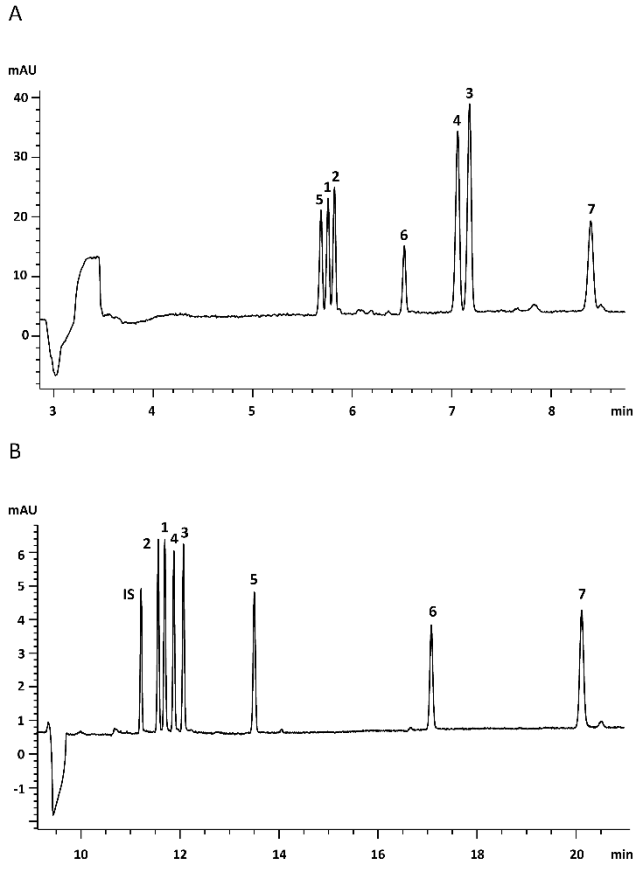
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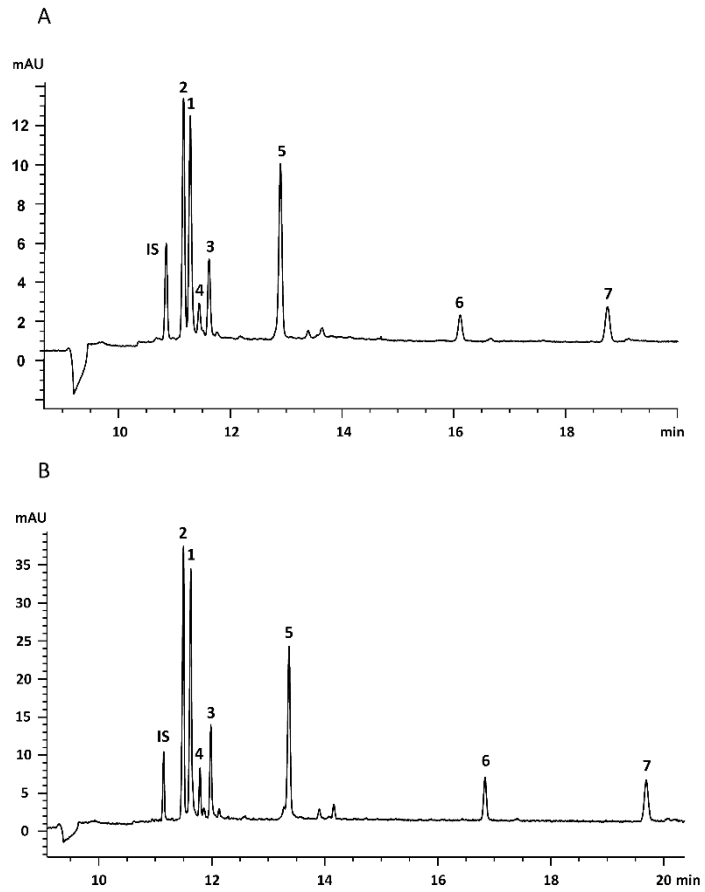
Figure 3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Supporting Information

Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex

Petra Riasová[1,2], Jaroslav Jenčo[3], David Moreno-González[1], Yvan Vander Heyden[2], Debby Mangelings[2], Miroslav Polášek[1], Pavel Jáč[1]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic

[2]Vrije Universiteit Brussel (VUB), Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling, Faculty of Medicine and Pharmacy, Laarbeeklaan 103, 1090 Jette, Brussels, Belgium

[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic

Corresponding author: Dr. Pavel Jáč, Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, CZ-500 05, Czech Republic

E-mail: jac_p9aa@faf.cuni.cz **Telephone number:** +420 495 067 504

Table S1: Optimization of SDS concentration of the MEKC method.

Capillary length	**BGE / E**	**SDS (mmol L^{-1})**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**
48.5/40 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, pH 9.0, 10% (v/v) MeOH / 515 V cm^{-1}	70	0.67	0	51
		140	1.26	0	81
		220	2.00	0	126

Table S2: Optimization of CD content.

Capillary length	**BGE / E**	**β-CD (mmol L^{-1})**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**
48.5/40 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, pH 9.0, 10% (v/v) MeOH / 515 V cm^{-1}	0.5	0	0.49	26
		1.0	0	0.63	26
		1.5	0	0.60	26
		2.0	0	0.58	26
		2.2	0	0.55	26
		2.5	0	0.52	26
		3.0	0	0.44	26
		10.0	0	0	29
		15.0	0	0	27
Capillary length	**BGE / E**	**2-HP-β-CD (mmol L^{-1})**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**
48.5/40 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, pH 9.0, 10% (v/v) MeOH / 515 V cm^{-1}	0.5	0	0.78	29
		1.0	0	0.86	29
		1.5	0	0.80	26
		2.0	0	0.63	26
		2.5	0	0.56	26
		3.0	0	0.43	26
		5.0	0	0	26

Table S3: Optimization of 2-HP-β-CD concentration in CD-MEKC mode.

Capillary length	**BGE / E**	**2-HP-β-CD (mmol L^{-1})**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**
48.5/40 cm, ⌀ 50 µm	140 mmol L^{-1} SDS, 100 mmol L^{-1} boric acid, pH 9.0, 10% (v/v) MeOH / 515 V cm^{-1}	1.5	0.94	0.61	87
		2.0	1.13	0.77	87
		2.5	1.18	0.87	87
		3.0	1.21	1.05	70
		5.0	1.22	1.40	85
		7.0	0.93	1.15	95
		10.0	0.92	1.55	95

Table S4: Optimization of MeOH content in CD-MEKC mode.

Capillary length	**BGE / E**	**Methanol % (v/v)**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**	**t_{mig} (min)***
48.5/40 cm, ⌀ 50 µm	140 mmol L^{-1} SDS, 100 mmol L^{-1} boric acid, 5 mmol L^{-1} 2-HP-β-CD, pH 9.0 / 515 V cm^{-1}	5	0.94	1.38	100	6.56
		10	1.33	1.62	90	8.50
		15	1.07	1.30	90	12.50

* migration time of the last analyte from the seven analysed substances

Table S5: Optimization of TM-β-CD content in EKC method.

Capillary length	**BGE / E**	**TM-β-CD (mmol L^{-1})**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**	**t_{mig} (min)***
48.5/40 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, pH 9.0, 10% (v/v) MeOH / 515 V cm^{-1}	1.0	0.94	0.97	31	6.70
		2.5	1.04	0.31	33	6.60
		5.0	1.40	2.05	32	6.72
		5.5	1.23	2.13	32	6.76
		6.0	1.27	2.06	32	6.68
		6.5	1.25	1.90	32	6.55
		7.0	1.19	1.78	32	6.51
		7.5	1.29	1.91	32	6.60
		10.0	1.11	1.82	31	6.56
		15.0	1.16	1.54	31	6.42

* migration time of the last analyte from the seven analysed substances

Table S6: Optimization of pH, MeOH content, and capillary length of EKC method.

Capillary length	**BGE / E**	**pH**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**	**t_{mig} (min)***
64.5/56 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, 5 mmol L^{-1} TM-β-CD, 10% (v/v) MeOH / 387.6 V cm^{-1}	8	0	1.31	10	8.69
		9	1.43	2.24	24	12.79
		10	1.61	3.28	46	20.02
Capillary length	**BGE / E**	**MeOH (v/v) %**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**	**t_{mig} (min)***
64.5/56 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, 5 mmol L^{-1} TM-β-CD, pH 9.0 / 387.6 V cm^{-1}	0	0.89	1.76	36	8.95
		5	1.18	2.00	34	11.35
		10	1.47	2.17	31	14.44
		15	1.60	2.08	25	17.22
		20	1.84	2.05	24	22.99
Capillary length	**BGE / E**	**TM-β-CD (mmol L^{-1})**	**Rs $_{SB\ A/B}$**	**Rs $_{ISB\ A/B}$**	**I (µA)**	**t_{mig} (min)***
80.5/72 cm, ⌀ 50 µm	100 mmol L^{-1} boric acid, 10% (v/v) MeOH, pH 9.0 / 310 V cm^{-1}	5	1.73	2.59	18	20.98

* migration time of the last analyte from the seven analysed substances

Table S7: Average tablet contents (n=3) of seven silymarin compounds in dietary supplements. The total amount is calculated as the sum of the six flavonolignans.

	Amount found in one tablet (mg) ± SD (n = 3)							
	CE							
	Silybin B	**Silybin A**	**Isosilybin B**	**Isosilybin A**	**Silychristin**	**Silydianin**	**Taxifolin**	**Total**
Preparation 1	18.3 ± 1.2	18.2 ± 0.9	2.6 ± 0.1	5.6 ± 0.3	16.5 ± 0.5	3.2 ± 0.1	4.0 ± 0.2	64.2 ± 2.9
Preparation 2	24.7 ± 1.1	22.6 ± 0.7	3.3 ± 0.1	7.2 ± 0.4	20.8 ± 0.8	6.1 ± 0.2	5.6 ± 0.3	84.7 ± 3.1
	HPLC							
	Silybin B	**Silybin A**	**Isosilybin B**	**Isosilybin A**	**Silychristin**	**Silydianin**	**Taxifolin**	**Total**
Preparation 1	22.3 ± 2.1	14.8 ± 1.4	1.9 ± 0.2	5.6 ± 0.6	18.2 ± 1.6	6.00 ± 0.6	3.8 ± 0.4	68.8 ± 6.5
Preparation 2	28.2 ± 3.5	18.5 ± 2.4	2.8 ± 0.4	6.9 ± 0.9	22.2 ± 2.8	9.2 ± 1.1	5.3 ± 0.6	87.9 ± 11.0

Table S8: Comparison of required and average found contents (n = 3) of six flavonolignans in the dietary supplements determined by (A) CE and (B) HPLC.

A

	Silybin A + Silybin B			Isosilybin A + Isosilybin B			Silychristin + Silydianin		
	Required[a)] amount (%)	**Found content (mg)**	**Found content (%)**	**Required[a)] amount (%)**	**Found content (mg)**	**Found content (%)**	**Required[a)] amount (%)**	**Found content (mg)**	**Found content (%)**
Preparation 1	40-65	36.5	56.8	10-20	8.1	12.6	20-45	19.7	30.6
Preparation 2	40-65	47.3	55.9	10-20	10.5	12.4	20-45	26.9	31.8

B

	Silybin A + Silybin B			Isosilybin A + Isosilybin B			Silychristin + Silydianin		
	Required[a)] amount (%)	**Found content (mg)**	**Found content (%)**	**Required[a)] amount (%)**	**Found content (mg)**	**Found content (%)**	**Required[a)] amount (%)**	**Found content (mg)**	**Found content (%)**
Preparation 1	40-65	37.1	53.9	10-20	7.5	10.9	20-45	24.2	35.2
Preparation 2	40-65	46.7	53.2	10-20	9.8	11.1	20-45	31.4	35.8

[a)]Ph. Eur. and USP

Table S9: HPLC method calibration curves parameters for individual compounds in the range 4-50 µg mL^{-1}.

Parameter	Silybin B	Silybin A	Isosilybin B	Isosilybin A	Silychristin	Silydianin	Taxifolin
Slope	26.4 ± 0.3	25.7 ± 0.2	25.5 ± 0.2	27.7 ± 0.20	19.7 ± 0.1	20.8 ± 0.2	29.6 ± 0.3
Intercept	-2.8 ± 7.8	-5.8 ± 6.5	2.5 ± 6.8	-4.8 ± 5.6	4.8 ± 4.0	-8.1 ± 6.6	-4.2 ± 8.0
Correlation coefficient (r)	0.9997	0.9998	0.9998	0.9999	0.9998	0.9997	0.9998

Table S10: Assessment of peak purity of all peaks for Preparation 2 (average from 12 electropherograms)

Compound	Peak purity	Threshold	No. of spectra
IS	995	976	20
SB B	991	978	39
SB A	990	974	41
ISB B*	980	863	12
ISB A	985	941	25
SCH	992	978	48
SDA	NED	-	-
TX*	978	895	20

NED: not enough data points for the evaluation of peak purity; *: In some electropherograms, insufficient number of data points available for the evaluation of peak purity: ISB B: average from 11 electropherograms, TX: average from 8 electropherograms.

Table S11: Comparison of the new CE-UV method for the separation of main flavonolignans with other reported methods.

Method	Detected compounds (in the order of the elution)	Range (µg mL^{-1})	LOQ (µg mL^{-1})	Precision RSD (%)	Time of separation (min)	Reference
EKC-UV	SB B, SB A, ISB B, ISB A, SCH, SDA, TX	2.5 – 50	≤ 2.1	≤ 6.6	25	Present work
UHPLC–IM–QTOF	TX, ISCH, SCH A, SCH B, SDA, SB A, SB B, cSB B, ISB A , ISB B, DHSB	0.05 – 5 0.05 - 1 (DHSB)	0.05	< 10	14.5	[1]
UHPLC-UV	TX, SCH, SDA, SB A, SB B, ISB A, ISB B	0.5 - 50	≤ 0.23	≤ 4.70	10.5	[2]
HPLC-UV	TX, ISCH, SCH, SDA, SB A, SB B, ISB A, ISB B	3 - 630	3	≤ 2.79	32	[3]
HPLC-UV	SCH, SDA, SB A, SB B, ISB A, ISB B	1.5 – 150 0.75 – 75 (ISB B, ISB A)	≤ 0.27	≤ 9.88	45	[4]
LC-MS	SCH, SDA, SB A, SB B, ISB A, ISB B	0.10 – 100 0.25 – 100 (SCH, SDA)	≤ 0.25*	≤ 12.4	25	[5]
CZE- UV	SDA, SCH, ISB, SB B, SB A	10 - 200	0.5	0.88	10	[6]
HPCE-UV	ISB, SB, SCH, TX, SDA	30 - 200	-	≤ 2.0**	9	[7]

IM – ion mobility, cSB B - 2,3-cis-silybin B, DHSB - 2,3-dehydrosilybin

*LLOQ for SD and SCH

**repeatability of peak-area ratios

Reference

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