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Determination of silymarine in the extract from the dried silybum marianum fruits by high performance liquid chromatography and capillary electrophoresis

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Abstract

Silybine (SBN), isosilybine (ISBN), silycristine (SCN), silydianine (SDN), and taxifoline (TXF) are the main active flavanoids, generally found in the dried fruits of silybum marianum. The concentrations of these compounds, excepted TXF, are all together usually expressed as silymarine content. In this paper the determination of the silymarine titre was made by high performance liquid chromatography (HPLC), and high performance capillary electrophoresis (HPCE). Two reversed stationary phases, RP-18 and RP-8, were observed comparing the resolutions of all considered flavanoids with each stationary phases. The HPCE was carried out considering the possible improvement in the resolution of SBN, CN, SDN and TXF using,8-cyclodextrines or organic modifier. The qualitative and quantitative data obtained by HPLC and HPCE were compared. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: High performance liquid chromatography; High performance capillary electrophoresis; Determination of silymarine

1. Introduction

A standardized extract, obtained from the dried fruits of silybum marianum, is on sale in Italy as antihepatotoxic drug. The main active substances among components of this extract, are five flavanoids (Fig. 1) like silybine (SBN), isosilybine (ISBN), silycristin (SCN) silydianin (SDN), and taxifoline (TXF). Theoretically SBN has 16 diastereoisomers, but in the nature only two of them are produced.

Generally the title of the silybum marianum extract is expressed as silymarine percentage and it corresponds to the sum of SBN, ISBN, SDN, and SCN concentrations. Therefore to determine sylimarine correctly it is necessary to use a selective and accurate analytical method.

The flavanoids of silymarine were previously analyzed by TLC [1,2], high performance liquid

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chromatography (HPLC) [3–5] and UV-Vis spectrophotometry. This last technique is proposed for the quantitative control of sylimarine in the monograph of the Italian Pharmacopea [6] related to silybum marianum. This spectrophotometric method is time consuming and shows a not satisfactory repeatability.

In this paper we propose a HPLC and a high performance capillary electrophoretic (HPCE) methods to determine the percentage of silymarine in the extract of dried silybum marianum fruits. The data obtained from the quantitative analyses of the same samples made by HPLC and HPCE were compared.

2. Experimental

2.1. Chemicals

All solvents and chemicals were of HPLC grade (Merck, Darmstadt, Germany). Silybine pure standard has been obtained from Carl RothKG (Karlsruhe, Germany) while standards of SBN, ISBN, SCN, SDN, and TXF were kindly supplied from IBI (laboratori Farmaceutici- Aprilia, Italy).

2.2. Apparatus

2.2.1. High performance capillary electrophoresis

The analyses of silybum marianum samples have been carried out with a spectraphoresis ultra apparatus (Spectra PHORESIS SN4000, Thermo Quest Italia, Fremont, CA) equipped with a vial server and a multiwavelength detector (W 3000). The instrument was controlled and the data were evaluated by a Pentium 133 with software tsp PC 1000 3.0.

2.2.2. High performance liquid chromatography

A Merck-Hitachi Series L 7000 chromatograph equipped with a Merck-Hitachi Series L 7450 photodiode array detector, have been used in the analyses made by HPLC. The chromatograph was controlled and the data evaluated by a computer Flyer Pentium, interface D 7000. Sample solutions were injected via a Rheodyne Model 7725 i valve using a 20μl sample loop.

2.3. Analysis conditions

2.3.1. High performance capillary electrophoresis

The separation of analyses was performed in an uncoated capillary having a total length of 43 cm (35 cm effective length, 50 micron I.D). A 100 mM borax solution, mixed with 100 mM boric acid until pH 9 and added with 15% methanol, was used as background electrolyte B.G.E.). The samples were injected for 2 s and the analyses were carried out at a λ value of 200 nm.

2.3.2. High performance liquid chromatography

The separation of Silybum Marianum flavanoids was carried out using two different stationary phases: a Purospher C18 (250×4mm I.D., 5 micon) (System (a)) and a Lichrosphere C8 (250 mm $\times$ 4 mm I.D, 5 micron) (System (b)) The mobile phases used were:

2.3.2.1. System (a). Water, acidified until pH 2.6 with 10% H_3PO_4, was mixed with acetonitrile in the ratio of 62:38. The elution has been made in isocratic mode at a flow rate of 1 ml min^{-1}.

2.3.2.2. System (b). Solvent A = Water acidified at pH 2.3 with 10% H_3PO_4 solution; Solvent B = acetonitrile; Solvent C = methanol. The separation of analyses was made using a gradient elusion as follow at a flow rate of l ml min^{-1}.

Time	% water	% acetonitrile	%methanol
0.0	63	15	22
7.5	63	15	22
8.5	40	20	40
15.0	40	20	40

The photodiode array detector conditions were:
λ value 289 nm.
Acquisition rate of spectra 1600 ms.
Spectral bandwidth for each channel 4 nm.
Wavelength range: 220–350 nm.

2.4. Extraction procedure from the dried fruits of silybum marianum

Five grams of finely powdered silybi mariani fructi were extracted with petroleum ether by a

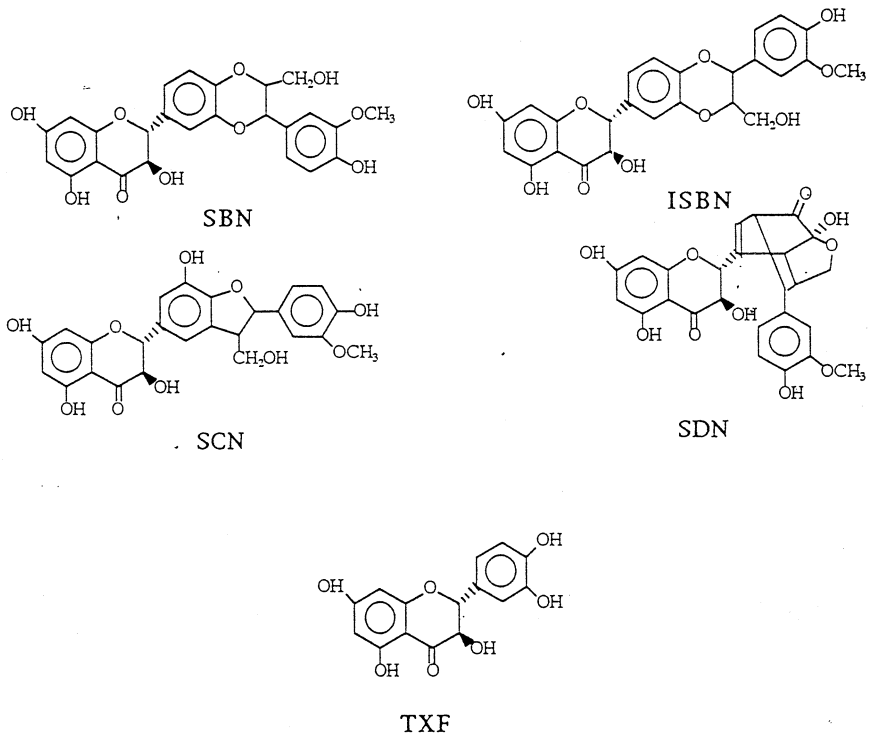

Fig. 1. Chemical structure of Silymarine flavanoids.

soxhelet apparatus for 4 h. This first extracted solution was discarded and the soxhelet apparatus dried with air. Then the drug was extracted again twice with methanol for 5 h and the methanolic solutions were evaporated to dryness on a rotary evaporator at about 50°C.

2.5. Working standards mixture

A working standard mixture containing SBN, ISBN, SCN, SDN, and TXF was used to know their chromatographic and electrophoretic behaviour in the analytical conditions described in Section 2.3.

2.6. Reference solution

A reference solution was freshly prepared exactly weighing an amount of about 20 mg SBN reference standard. SBN was transferred in a 100 ml volumetric flask and solubilized to volume with methanol. By opportune dilution of this methanolic solution a series of solutions at different concentrations were prepared and used to verity the linearity of calibration curves whether in HPLC or in HPCE.

2.6.1. High performance liquid chromatography

The reference standard solution of SBN, prepared as above described, was used as external standard. The range of solution concentrations, obtained by opportune dilution of reference solution, was between 0.010 and 0.20 mg ml^{-1}. The data obtained from the analysis of each solution allowed to plot a calibration curve showing a good linearity (correlation coefficient = 0.9998).

2.6.2. High performance capillary electrophoresis

By appropriate dilution of the SBN reference standard solution, a series of solutions having a concentration range between 0.03 and 0.2 mg ml^{-1} were obtained. Since the quantitative analysis by HPCE needs internal standard, all solutions were added with the same amount of quercetin (0.075 mg ml^{-1}).

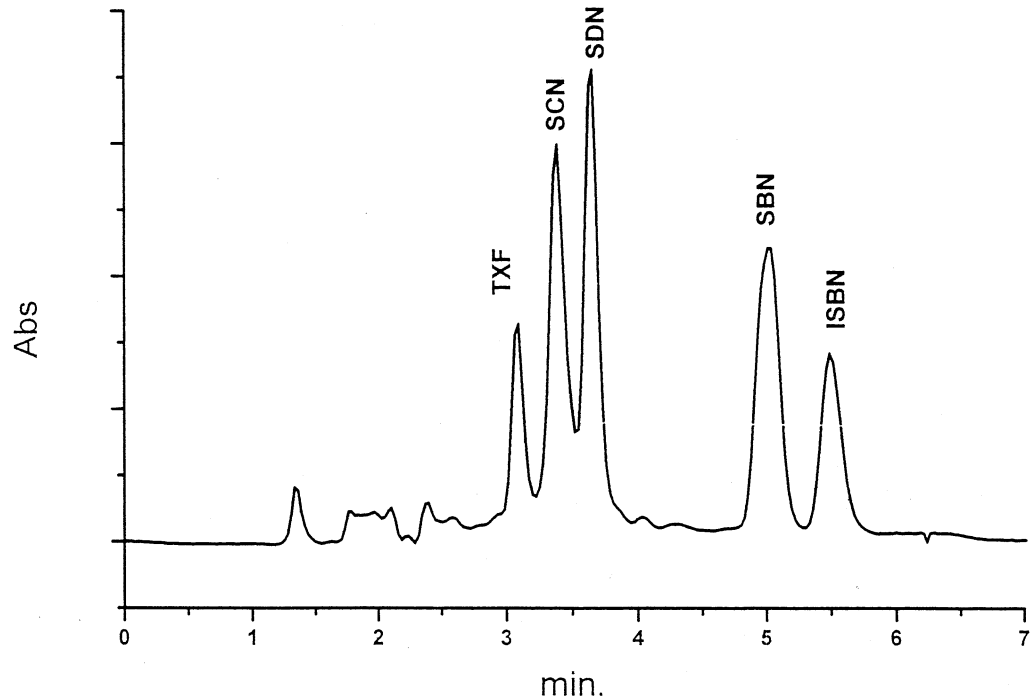

Fig. 2. Chromatogram obtained from the extract with a RP-C18 stationary phase. Mobile phase: as described in Section 2.3.

2.7. Sample solutions

The samples examined were two extraction residues, obtained from two different dried fruits of silybum marianum (sample a and b) and two lots of silymarine bulk material (sample c (lot 30413) and sample d (lot 60830)), used for the preparation of a pharmaceutical form in tablets.

The four samples, exactly weighed, were separately dissolved in methanol and the solutions transferred in distinguished 100 ml volumetric flasks carring upper to mark with the same solvent. Using a tared pipette 10 ml of each solution were separately transferred in volumetric flasks and diluted to 50 ml. The final concentration of methanolic solutions, filtered by a 0.45 μm membrane, was about 0.2–0.5 mg ml^{-1}.

20 μl of each solution, having the concentration above mentioned, were used for the quantitative determination by HPLC. The sample solutions, analyzed by HPCE, were introduced in the apparatus by hydrodinamic mode for 2 s (about 7 m).

3. Results and discussion

The extracts obtained as described in experimental section were analyzed by HPLC and HPCE comparing the suitability of both techniques.

3.1. High performance liquid chromatography

A RP-18 and a RP-8 stationary phases were tested for the separation of SBN, ISBN, SCN, SDN, and TXF mixture. The RP-18 column allowed a good separation among TXF, SBN, and ISBN, while the peaks related to SDN and SCN were not base line resolved (Fig. 2). The increasing of the water percentage in the mobile phase didn't improved the separation of SDN from SCN but allowed the separation of two diastereomers of SBN.

The use of the more polar phase RP-8 with a suitable gradient elution improved the resolution of all peaks (Fig. 3). This second chromatographic system resolved base line SDN, SBN, ISBN,

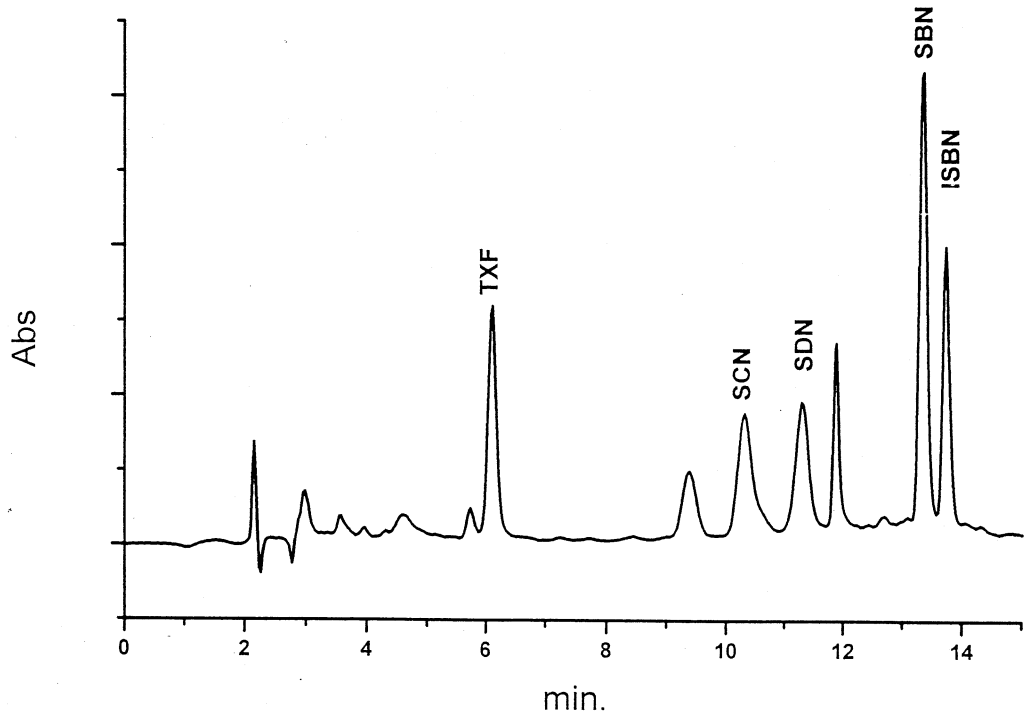

Fig. 3. Chromatogram obtained from the extract with a RP-C8 stationary phase. Mobile phase: gradient as described in Section 2.3.

Table 1
All ammounts are expressed in mg[a]

	HPLC				CE			
Samples	SBN	SCR	SDN	TXF	SBN	SCR	SDN	TXF
Sample a	58.00	33.00	37.50	16.50	59.10	37.50	30.05	16.50
Sample b	65.00	34.50	40.50	17.75	66.50	35.5	38.64	18.80
Sample c (19.4 mg of lot 30413)	8.4	2.35	1.25	0.55	9.11	2.66	1.36	1.11
Sample d (16 mg of lot 60830)	7.15	2.6	Not detected	0.40	8.21	3.3	Not detected	0.65

[a] The amounts of flavanoids found in the samples a and b have been referred to 5 g of silybum marianum fruits.

SCN, and TXF, and two non identified peaks which were before co-eluted with SCN and SDN, respectively. This chromatographic method allows to obtain a more precise qualitative and quantitative analytical profile. Actually the good separation of all compounds allowed the purity control of each peak, made by diode array detector, the plotting of UV spectra, useful for the peak identification and a more correct quantitation. The use of RP-8 stationary phase didn't allowed the resolution of the two SBN diastereomers. Being SBN used as external standard in the quantitative analysis it was better to have only one peak in place of the peaks of resolved diastereomers.

The quantitative data obtained from the HPLC samples analysis have been summarized in Table 1.

3.2. High performance capillary electrophoresis

The analysis of SBN, ISBN, SCN, SDN, and TXF by HPCE was carried out on an uncoated

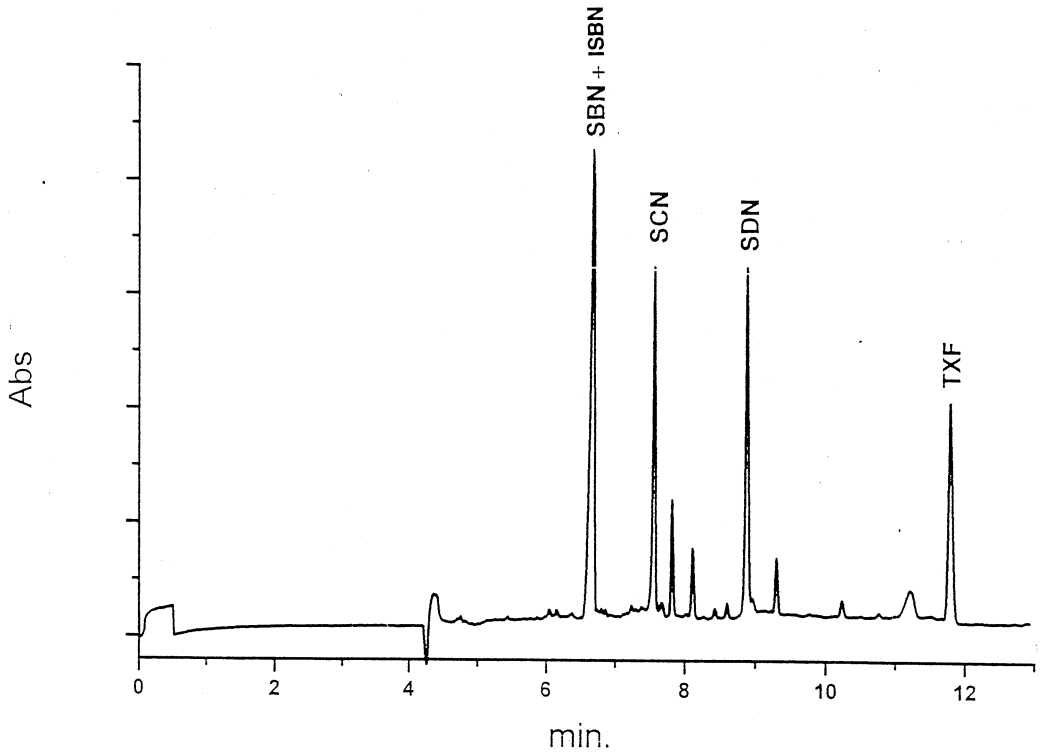

Fig. 4. Electropherogram obtained from the extract using an uncoated capillary 43 cm (effective length 35 cm) 0.05 mm ID; BGE:100 mM borax solution mixed with 100 mM boric acid containing 5% of methanol, pH 9; sample introduction by hydrodynamic mode for 2 s; applied voltage 20 KV 60 μA.

capillary filled with a borate buffer solution at pH 9, added with 15% methanol to change the background electrolyte viscosity. At this pH value the considered flavanoids and acidic substances, because of the presence of many phenolic groups in their structure, were negatively charged. Actually, as it can be seen in Fig. 4, SBN, SCN, SDN, and TXF cross the detection window after electroosmotic flow, all very well separated. In these conditions ISBN was co-eluted together with SBN, but adding a12 mM dimethyl β-cyclodextrines solution to the running buffer the separation of SBN from ISBN (Fig. 5) was obtained. Further, the addition of dimethyl β-cyclodextrines increased significantly the mobility of TXF while decreased the mobility of SDN.

We carried out the quantitative analysis using an internal standard and the peak areas normalization. The internal standardization was used to eliminate the possible differences in the amount injected, increasing the repeatability. The peak area normalization was made to eliminate the probable differences in the peak velocities [7–9]. Actually the more mobile analytes migrate more rapidly along the capillary and cross the detector more quickly than the less mobile species. Being the peak area related whether to the solute concentration (peak height) or the residence time of the compound in the detection window (peak width), the normalization is obtained dividing the area of each peak by corresponding migration time.

In our test the quantitative determination was carried out using quercetine as internal standard. Quercetine is a flavanoid contained in many plants, but never found in the samples examined.

The calibration curve was plotted analyzing solutions at different concentrations of SBN, all

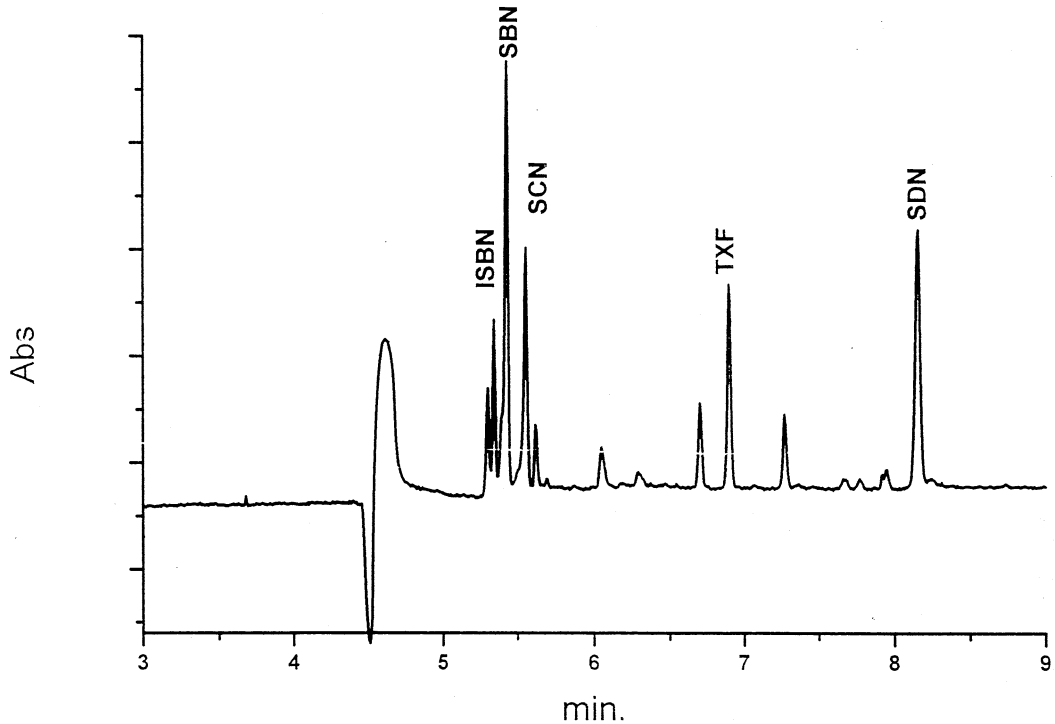

Fig. 5. Electropherogram obtained from the extract using an uncoated capillary 43 cm (effective length 35 cm) 0.05mm ID; BGE a 100 mM borax solution mixed with 100 mM boric acid, pH 9, added with 12 mM dimethyl β-cyclodex trins and 15% of methanol; sample introduction by hydrodynamic mode for 2 s; applied voltage 20 KV (constant), 60 μA; temperature of the capillary tube 20°C.

added with a fixed amount of quercetine. The linearity, obtained by plotting the peak-area ratio vs. concentration for each substance analyzed, gave a correlation coefficients between 0.9988 and 0.9994.

The quantitative data obtained from the samples analysis by HPCE have been reported in Table 1.

Table 2
All results are expressed in % w/w

Samples	silymarine by HPLC (%)	silymarine by CE (%)
Sample a	2.56	2.53
Sample b	2.8	2.81
Sample c (lot 30413)	61.91	67.68
Sample d (lot 60830)	60.9	71.9

The repeatability (relative standard deviation) of the proposed methods, on the basis of peak-area ratios for six replicate injections of each sample, was 0.84–1.35% for HPLC determinations and 0.95–2% for HPCE. Also the relative standard deviation of the retention or migration times of each peak for six replicate injections was tested. It was 0.54% for HPLC and 2.24% for HPCE.

At least Table 2 reports the concentrations of all flavanoids found in the samples, examined by HPLC or HPCE, expressed as percentage of silymarine. In samples a and b the silymarine % has been referred to the weight of the dried fruits undergone to the extraction while in the sample c and d has been related to the bulk material sample weights.

4. Conclusions

The determination of sylimarine percentage in the dried Silybum Marianum fruits made by HPLC and HPCE gave comparable results. HPLC allowed the good separation between SBN and ISBN, while not a similar behaviour was obtained by HPCE. Nevertheless HPCE allowed a separation among SBN, SCS, SDN, and TXF by far the best compared to HPLC.

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