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SEPARATION OF BACOSIDE A_3 AND BACOPASIDE II, MAJOR TRITERPENOID SAPONINS IN *Bacopa monnieri*, BY HPTLC AND SFC. APPLICATION OF SFC IN IMPLEMENTATION OF UNIFORM DESIGN FOR HERBAL DRUG STANDARDIZATION, WITH THERMODYNAMIC STUDY

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SUMMARY

Development, optimization, and validation of new analytical methods for standardization of bacoside A_3 and bacopaside II, the major triterpenoid saponins present in *Bacopa monnieri* extract, are needed to improve the quality assurance of derived extracts and phytomedicines. Two chromatographic methods are described for evaluation of the quality of *Bacopa monnieri* extract and its commercial formulations. The first is reversed-phase high-performance thin-layer chromatography (RP-HPTLC), the second is packed column supercritical-fluid chromatography with photodiode-array detection (PC–SFC–DAD).

SFC conditions were optimized by uniform design. The effect of temperature on the separation of the saponins was studied in detail. The Van't Hoff plots for retention and selectivity were found to be linear. To obtain a better understanding of the different separations, the temperature dependence was studied to determine the thermodynamic data $\Delta H°$, $\Delta S°$, $\Delta\Delta H°$ and $\Delta\Delta S°$. These data revealed that separation of bacoside A_3 was enthalpically favoured in the range of temperatures investigated whereas entropy-controlled separation was observed for bacopaside II.

Both methods were validated for precision, robustness, recovery, and limits of detection and quantitation. Analysis of variance (ANOVA) and Student's *t*-test were used to correlate results from quantitative determination of the markers by RP-HPTLC and PC-SFC–DAD.

INTRODUCTION

The use of medicinal plants in both crude and prepared forms has increased substantially. Use of chromatography for standardization of plant products was introduced by the WHO and is accepted as a strategy for identification and evaluation of the quality of plant medicines [1–3]. The amounts of the same constituent in different samples of the same medicine may vary substantially, depending on geographic origin, climate, soil, harvest season, processing techniques, and other factors [4–7]. It is a fact that sufficient bioactive phytochemical content is crucial for therapeutic effect. Quality control of herbal medicines is, therefore, highly desired to ensure their authenticity, stability, and consistency.

A milestone in the elucidation of the pharmacologically active principles in *Bacopa monnieri* or brahmi was achieved by Chatterji and coworkers [8–10]. Bacoside A_3 and bacopaside II, the main triterpenoid saponins now regarded as responsible for the characteristic neuropharmacological effects of the plant, were obtained in the crystalline form from the whole plant. The saponins differ only in the nature of the sugar units in the glycosidic chain and the position of the olefinic side-chain in the aglycone (Figs 1a and 1b).

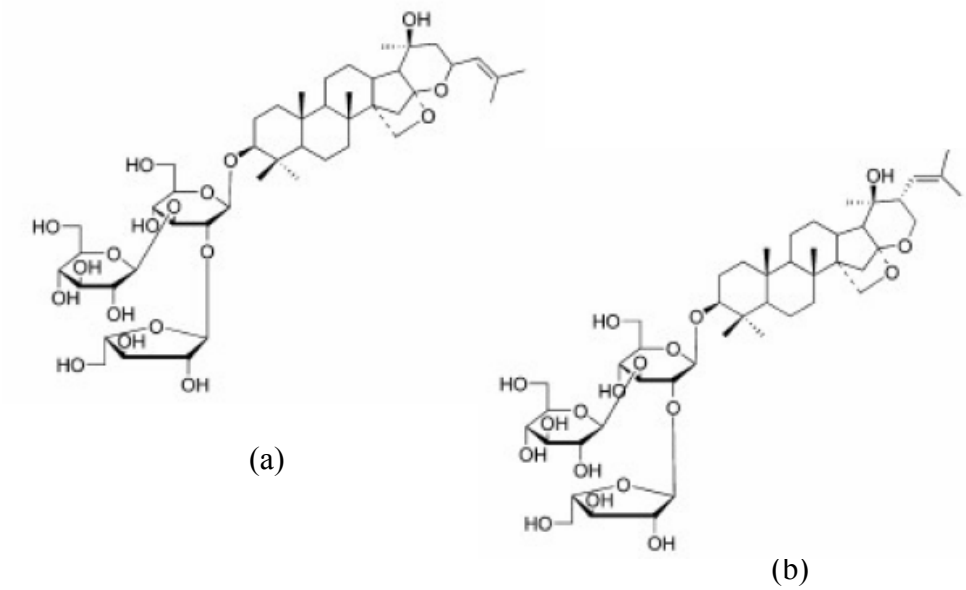

Fig. 1

The chemical structures of bacoside A_3 (a) and bacopaside II (b)

Roodenrys et al. has reported the chronic effects of brahmi on human memory [11]. Rastogi et al. isolated and characterized a new triterpenoid saponin, bacoside A_3, a constituent of bacosides, from the saponin mixture obtained from *Bacopa monnieri* [12]. Pal et al. performed quantitative analysis of bacoside by HPLC [13]. Chakravarty et al. isolated two saponins, bacopasides I and II, from *Bacopa monnieri* [14]. Deepak et al. performed quantitative analysis of the major saponin mixture bacoside A from *Bacopa monnieri* by HPLC [15]. Kawai et al. performed acid hydrolysis of bacoside A from *Bacopa monnieri* and obtained ebelin lactone and bacogenin-A_1 [16].

Methods described in the literature for analysis of bacosides are mainly based on UV spectroscopy [17,18], thin-layer chromatography [19], and HPLC [20–22]. Sivaramakrishna et al. isolated two triterpenoid glycosides and ten known saponins from *Bacopa monnieri*. The structures of the triterpenoid glycosides were elucidated as 3-*O*-[β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl]jujubogenin and 3-*O*-[β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl]pseudojujubogenin by use of high-resolution NMR spectral data and chemical correlation [23]. Ganzera et al. described the first analytical procedure enabling analysis of the individual bioactive saponins (bacosides) in *Bacopa monnieri*. The main components were either bacoside A_3 or bacopaside II; bacopasides IV and V were present at lower concentrations [22]. LC–MS has recently been found to be a valuable technique for providing molecular mass and structural information about these compounds, but the equipment is expensive and not readily available to most quality-control laboratories. Liquid chromatography is not the best system for quantification of these compounds, because most solvents absorb strongly at the short wavelengths required for sensitive detection. These methods also require lengthy sample preparation and have rather high limits of detection. Carbon dioxide under supercritical conditions is an excellent solvent for a variety of organic molecules which absorb at short UV wavelengths.

HPTLC is becoming a routine analytical technique because of its advantages of low operating cost, high sample throughput, simplicity, and speed, the need for minimum sample clean up, reproducibility, accuracy, reliability, and robustness [24–26].

Among published methods there are no integrated experimental design based studies which include development, optimization, and validation of the method. In this paper we describe strategies for method development, optimization, and validation of RP-HPTLC and PC-SFC–DAD

methods for standardization of *Bacopa monnieri* extracts. Commercial formulations containing the major bioactive constituents of these extracts (with bacoside A_3 and bacopaside II as analytical markers) were also quantified. Controllable conditions and other factors which must be optimized were selected for PC-SFC–DAD. Optimization was performed by use of a uniform design, which is very useful when many levels must be evaluated. Use of this approach is extremely beneficial in the context of plant analysis when the components have fairly similar chemical structures and their separation is still a challenging task for the pharmaceutical analyst. The last stage was method validation of injection precision, repeatability, reproducibility, and sample stability, as recommended in the ICH guidelines [27–29]. An effort was also made to evaluate, thermodynamically, the temperature dependence of the behaviour of the saponins in PC-SFC–DAD.

EXPERIMENTAL

Pharmaceutical grade Bacoside A_3 and bacopaside II were kindly supplied as gifts by Laila Impex, Vijaywada, India; they were certified to contain 97 and 98% (*w*/*w*), respectively, on a dry-weight basis, and were used without further purification. *Bacopa monnieri* extract and capsules were obtained commercially from the local market. All chemicals and reagents were of HPLC grade and were purchased from Merck Chemicals, India. Before use methanol was filtered through a 0.45-μm filter (Millipore, Milford, MA, USA) and degassed by sonication. Carbon dioxide, 99.9% pure for SFE/SFC, was obtained from Bombay Carbon Dioxide, Mumbai, India.

HPTLC was performed on 20 cm × 10 cm aluminium-backed HPTLC plates coated with 200-μm layers of RP-18F_{254} silica gel (E. Merck, Darmstadt, Germany; supplied by Anchrom Technologists, Mumbai, India). Before use the plates were prewashed with methanol and activated at 60°C for 5 min. Samples were applied as 6 mm wide bands, 6 mm apart, by the spray-on technique, by means of a Camag (Switzerland) Linomat IV sample applicator fitted with a 100-μL syringe (Hamilton, Bonaduz, Switzerland). A constant application rate of 0.1 μL s^{-1} was used. Plates were developed to a distance of 9 cm, in the dark, with toluene–methanol–ethyl acetate, 7.5:2.5:2.0 (*v*/*v*), as mobile phase. The volume of mobile phase was 15 mL. Before development the chamber was saturated with mobile phase for 30 min at room temperature (25 ± 2°C). Chromatography was performed in a controlled humidity chamber; relative humidity was fixed at 60 ± 5%. These conditions resulted in good resolution. Densitometric

scanning was performed with a Camag TLC scanner III in reflectance–absorbance mode at 344 nm, under control of CATS software (V 3.15, Camag). The slit dimensions were 5 mm × 0.45 mm and the scanning speed was 10 mm s^{-1}.

SFC was performed with a Jasco (Japan) 1500-series supercritical-fluid chromatograph configured for dynamic mixing with a Jasco-PU 2080 two-pump system. The instrument incorporates a facility for on-line addition of organic modifier to the supercritical mobile phase. The apparatus was capable of pressures in the range 7 to 45 MPa and a Jasco CO-2065 series oven enabled use of column temperatures in the range 35 to 80°C. A Jasco BP-1580-81 backpressure regulator, which enabled independent pressure control, maintained system pressure electronically. Compounds were separated on a 250 mm × 4.6 mm i.d., 5.0 μm particle, Finepak SIL-5, C-18 column (Jasco). A model-7125 Rheodyne injector with a 20-μL external loop was used to introduce sample to the column. Compounds were detected with a Jasco MD-2010 photodiode array detector with a 16-μL high-pressure flow cell (10 mm path length). Borwin chromatography software was used to record peaks and for data integration. A Fourtech (Mumbai, India) Cryostat bath was used for liquefaction of the gas.

Optimization of PC-SFC–DAD

A feasibly wide range of experimental conditions was chosen for each factor. Column temperature, pressure, and modifier concentration were recognized as important conditions affecting retention, selectivity, and column stability [30]. Uniform design is an experimental design, proposed by Fang and Wang in the 1980s and based on a quasi-Monte Carlo, or number-theoretical, method which allocates experimental points uniformly scattered on the domain [31,32]. The most widely used structure, the U-type design, which provides an *n*-run experimental design for '*s*' factors each having *n* levels, is denoted $U_n(n^s)$.

Preparation of Standard Solutions and Calibration Graphs

Stock standard solution was prepared by dissolving 5.0 mg bacoside A_3 and bacopaside II in 10 mL methanol (500 μg mL^{-1}). For densitometric RP-HPTLC standard solutions were prepared by dilution of the stock solution with methanol to furnish the concentration ranges 5.0–75.0 μg μL^{-1} and 5.0–80.0 μg μL^{-1} for bacoside A_3 and bacopaside II, respectively. For PC-SFC–DAD the standard solutions were prepared by dilution of the stock solution with methanol to furnish the concentration ranges 10.0–

120.0 μg mL^{-1} and 10.0-150.0 μg mL^{-1} for bacoside A_3 and bacopaside II, respectively.

Method Validation

Precision

Intra-day and inter-day variation for determination of bacoside A_3 and bacopaside II were determined at three different concentrations 15.0, 20.0, and 25.0 μg $band^{-1}$ and 20.0, 40.0, and 60.0 μg mL^{-1} for RP-HPTLC and PC-SFC, respectively.

Robustness

Mobile phases of different composition, for example toluene–methanol–ethyl acetate, 7.4:2.6:2.0 (*v*/*v*), 7.6:2.4:2.0 (*v*/*v*), 7.5:2.4:2.1 (*v*/*v*), 7.5:2.6:1.9 (*v*/*v*), 7.4:2.5:2.1 (*v*/*v*), and 7.6:2.5:1.9 (*v*/*v*) were investigated by developing chromatograms. The amount of mobile phase, temperature, and relative humidity were varied in the range ±5%. Plates were prewashed with methanol and activated at 110 ± 5°C for 5, 10, and 15 min before chromatography.

To evaluate the robustness of PC-SFC, pressure, the flow rate of supercritical carbon dioxide, amount of modifier, and column temperature were deliberately varied.

Limits of Detection and Quantitation

LOD and LOQ were determined experimentally by diluting known concentrations of bacoside A_3 and bacopaside II until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Specificity

The specificity of the RP-HPTLC method was ascertained by analysis of drug standards and a sample. The identities of the bands of bacoside A_3 and bacopaside II in the chromatogram obtained from the sample were confirmed by comparison of R_F values and spectra of the bands with those of standards.

The specificity of the PC-SFC method was determined by complete separation of bacoside A_3 and bacopaside II and other unknown components and by measurement and comparison of properties such as retention time (t_R), capacity factor (k), tailing or asymmetry factor (T), etc.

Recovery

For both methods recovery was determined by applying the method to a drug sample to which known amounts of bacoside A_3 and bacopaside II corresponding to 80, 100, and 120% of the label claim had been added (standard addition method).

Estimation of Bacoside A_3 and Bacopaside II from Herbal Extracts

To determine the amounts of bacoside A_3 and bacopaside II in extracts from different sources, 250 mg was transferred to a 100-mL volumetric flask containing 50 mL methanol, sonicated for 30 min, and diluted to 100 mL with methanol to furnish a final concentration of 2500 μg mL^{-1}. The resulting solution was centrifuged at 3000 rpm for 15 min and the supernatant was analysed for drug content. Different amounts (6, 10, and 24 μL) of sample solution were applied to plates six times to furnish amounts of 15.0, 25.0, and 60.0 μg $band^{-1}$ for bacoside A_3 and bacopaside II. Triplicate 20-μL volumes of sample solution (100 μg mL^{-1}) were analysed by SFC, six times, under the optimized conditions.

Analysis of a Commercial Formulation

To determine the amount of bacoside A_3 and bacopaside II in capsules (label claim 250 mg bacoside extract per capsule) the contents of twenty capsules were weighed, the mean weight in one capsule was determined, and the contents were finely powdered. The weight of powder equivalent to capsule content was transferred into a 500 ml volumetric flask containing 350 ml methanol, sonicated for 30 min and diluted to 500 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 15 min and supernatant was analyzed for drug content after making suitable dilutions of 100.0 mg mL^{-1} (for SFC) and 5.0 mg mL^{-1} (for HPTLC) and filtering through Watman paper No. 41. Twelve microlitres of the filtered solution (60.0 μg $band^{-1}$) was applied on the TLC plate followed by development and scanning and 100 μg mL^{-1} was analysed by SFC, six times, under the optimized conditions.

Effect of Temperature on Retention and Selectivity

The selectivity (α), i.e. the ratio of compound capacity factors, can be expressed on the basis of the van't Hoff equation [33] as:

$$\ln \alpha = -\Delta\Delta H^\circ/RT + \Delta\Delta S^\circ/R$$

where $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ are, respectively, the differences in the enthalpy

and entropy changes for the compounds, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), and T is the absolute temperature. Retention factors were calculated, with concomitant changes in molar enthalpy, to characterize the thermodynamic behaviour in the temperature range 303 to 328 K, the average pressure range 20 to 30 MPa, and for carbon dioxide mobile phases containing from 13 to 26% (*v*/*v*) methanol.

RESULTS AND DISCUSSION

Optimization of Procedures

RP-HPTLC–Densitometry

After several trials toluene–methanol–ethyl acetate, 7.5: 2.5: 2.0 (*v*/*v*), was found to give dense, compact bands, with typical peak shape,

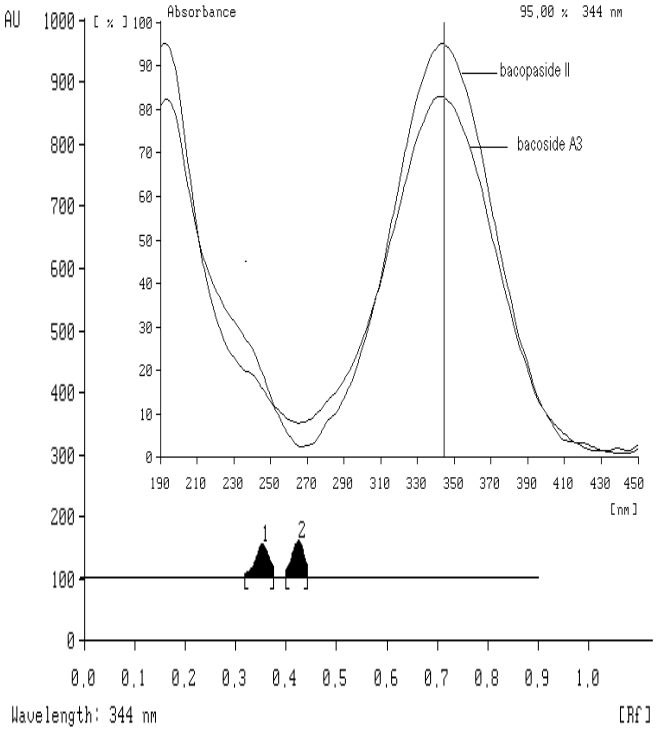

Fig. 2

Densitogram obtained, at 344 nm, from a mixture of standards of bacoside A_3 (5.0 µg $band^{-1}$; peak 1, R_F 0.34 ± 0.06) and bacopaside II (10.0 µg $band^{-1}$; peak 2, R_F 0.42 ± 0.05), and their overlain spectra from 190 to 450 nm

for bacoside A_3 and bacopaside II, and for other, unidentified, components, with excellent resolution. The peaks were symmetrical and no tailing was observed when plates were scanned at 344 nm (Fig. 2). Well-defined bands were obtained when the plate was activated at 60°C for 5 min and the chamber was saturated with mobile phase for 30 min at room temperature.

PC-SFC

The C_{18} reversed-phase column gave excellent chromatographic performance for the main components, with good separation between bacoside A_3 and bacopaside II (Fig. 3). Methanol was chosen as the modifier because the solutes were soluble in this solvent and could not be eluted with pure supercritical carbon dioxide [34]. Initially temperature and pressure were optimized. The optimization region selected was between 18–30 MPa pressure and 27 to 45°C (detection was at 210 nm). Two conditions, gradient time t_G and the concentration of modifier at the beginning of the gradient (B%), were chosen for optimization of the gradient. The optimization region selected was 6.0–9.0 min for t_G and 15.0 to 25.0% for B.

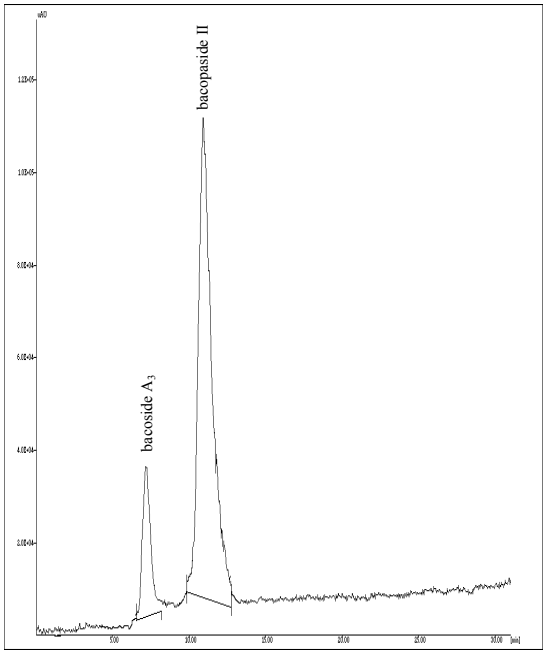

Fig. 3

Supercritical-fluid chromatogram obtained, at 210 nm, from a mixed solution (100.0 µg mL^{-1}) of bacoside A_3 (t_R 7.11 ± 0.05) and bacopaside II (t_R 11.3 ± 0.06) standards

The final concentration of B was set at 6% of the initial concentration of modifier, to ensure that every solute eluted in one run. Using the uniform table U_7 (7^2), seven experiments were designed (Table I). As shown in Fig. 4, the designed points are scattered uniformly in the region of the variables.

Table I

Uniform design with seven experiments for two factors (pressure and temperature) at seven levels U_7 (7^2)

No.	Factor	
	Pressure (X_1)	Temperature (X_2)
1	4 (24 MPa)	4 (36°C)
2	3 (22 MPa)	7 (45°C)
3	5 (26 MPa)	1 (27°C)
4	2 (20 MPa)	2 (30°C)
5	1 (18 MPa)	5 (39°C)
6	7 (30 MPa)	3 (33°C)
7	6 (28 MPa)	6 (42°C)

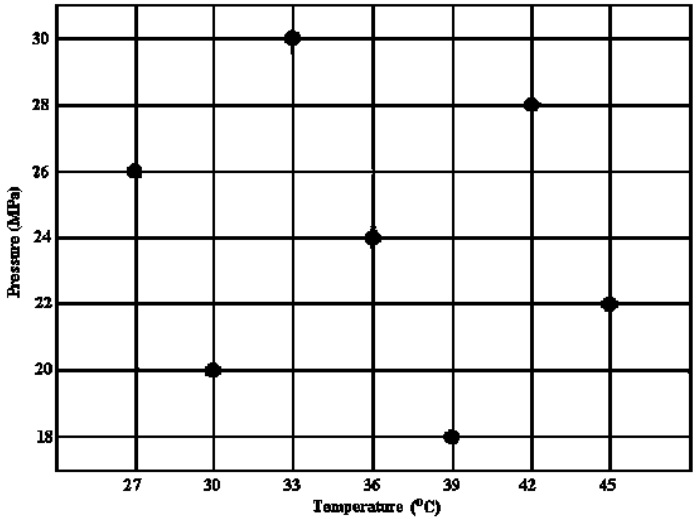

Fig. 4

Uniform design setting of the seven experimental runs in a U_7 (7^2) design using pressure and temperatures as variables

Evaluation criteria based on R_S, the resolution between two neighbouring peaks, for example the sum of all the resolution values (ΣR_S), were used for evaluation of the separation quality. A normalized resolution factor was defined as:

$$r^* = \prod_{i=0}^{n-1} \left(\frac{R_{si,i+1}}{\overline{R}_S} \right)$$

to achieve uniform distribution of detected peaks. For r^*, because of normalization, a very short chromatogram with all components evenly distributed will still be heavily favoured, even if the maximum observed resolution is small. A hierarchical chromatographic response function (HCRF) is suitable for this purpose:

$$\text{HCRF} = 1{,}000{,}000n + 100{,}000R_{\min} + (t_m - t_l)$$

where n is the number of peaks in the chromatogram, $R_{\min}$ is the resolution of the least separated pair of peaks, and t_m and t_l are, respectively, the maximum analysis time (30 min was chosen in this study) and the retention time of the last peak. From the HCRF output value the number of peaks, the worst resolution, and the analysis time are immediately apparent. The values of (ΣR_S), the number of theoretical plates (N), selectivity (α), r^*, and HCRF of the uniform design results are shown in Tables II and III. From these results the conditions for experiment No. 1 were selected in both designs. Therefore pressure of 24 MPa, a column temperature of 36°C, and linear gradient elution with modifier (methanol) content from 20 to 26% after 7.5 min and maintained for up to 30 min were chosen as the optimum conditions.

Table II

Results from uniform design for two factors, pressure and temperature, in PC-SFC–DAD

No.	Pressure (MPa)	Temperature (°C)	ΣR_S	ΣTp	r^* ($\times 10^3$)	$\Sigma\alpha$	HCRF
1	24	36	24.23	43,616	11.01	12.48	11,200,017.13
2	22	45	12.79	10,426	8.04	9.36	8,100,014.71
3	26	27	20.37	18,401	7.98	10.01	8,127,017.12
4	20	30	22.19	19,713	9.99	10.93	10,130,014.68
5	18	39	18.47	8,426	7.99	9.18	8,115,014.21
6	30	33	16.21	14,230	9.00	10.28	9,120,019.31
7	28	42	17.75	13,265	9.97	10.42	10,117,017.94

Table III

Results from uniform design for gradient elution in PC-SFC–DAD

No.	Modifier (%)	Time (min)	ΣR_S	ΣTp	r^* ($\times 10^3$)	$\Sigma\alpha$	HCRF
1	20.00	7.50	20.31	44,786	10.00	11.31	10,225,017.49
2	18.37	6.00	15.39	11,139	9.00	10.27	9,113,016.0
3	21.57	9.00	15.25	10,389	9.02	9.43	9,092,017.0
4	16.67	8.50	12.81	13,954	9.02	10.15	9,086,012.0
5	14.89	7.00	11.97	15,042	7.00	8.63	7,173,015.79
6	24.53	8.00	12.48	9,713	8.00	8.68	8,092,022.10
7	23.08	6.50	12.50	9,895	8.99	9.84	9,095,021.25

Linearity

Absorption patterns in HPTLC are described by the Kubelka–Munk model. Linear regression revealed good linear relationships for bacoside A_3 and bacopaside II in both RP-HPTLC and PC-SFC:

bacoside A_3, RP-HPTLC

$r^2 = 0.9989 \pm 1.6$, slope = 0.14 ± 0.02, intercept = 1.07 ± 0.12

bacopaside II, RP-HPTLC

$r^2 = 0.9980 \pm 1.7$, slope = 0.25 ± 0.06, intercept = 1.09 ± 0.14

bacoside A_3, PC-SFC

$r^2 = 0.9997 \pm 1.34$, slope = 0.48 ± 0.12, intercept = 1.02 ± 0.09

bacopaside II, PC-SFC

$r^2 = 0.9992 \pm 1.21$, slope = 0.57 ± 0.15, intercept = 1.16 ± 0.27

Precision

The repeatability of sample application and peak area measurement in HPTLC, expressed as %RSD, was 1.57 and 1.84, respectively, for bacoside A_3 and 1.34 and 1.61, respectively, for bacopaside II; %RSD for intra-day and inter-day variation were 1.31 and 1.64, respectively, for bacoside A_3 and 1.73 and 1.97, respectively, for bacopaside II. For PC-SFC intra-day and inter-day precision were 1.58 and 1.77, respectively, for bacoside A_3 and 1.86 and 1.92, respectively, for bacopaside II.

Robustness of the Method

In RP-HPTLC the standard deviation of peak areas was calculated for each condition and %RSD was found to be less than 2%. These low values of %RSD are indicative of the robustness of the method. The results

presented in Table IV for PC-SFC indicate that results were unaffected by small variation of the conditions selected – peak area and retention time differences were insignificant.

Table IV

Evaluation of robustness for PC-SFC (n = 6)[a]

Chromatographic changes		Chromatographic figures of merit							
		For bacoside A_3				For bacopaside II			
Factor[b]	Level	t_r[c]	k[d]	T[e]	η[f]	t_r[c]	k[d]	T[e]	η[f]
A: Pressure (MPa)									
24.50	−1	7.12	2.95	0.85	9210	11.54	5.30	0.96	7315
25.00	0	7.10	2.93	0.83	9140	11.50	5.27	0.94	7296
26.50	1	7.08	2.90	0.80	9073	11.47	5.24	0.91	7184
Mean ± SD (n = 6)		7.10 ± 0.02	2.93 ± 0.03	0.83 ± 0.03	9141 ± 0.75	11.50 ± 0.04	5.27 ± 0.03	0.94 ± 0.03	7205 ± 1.15
B: Flow rate of CO_2 (mL min^{-1})									
1.90	−1	7.15	2.96	0.86	9080	11.55	5.31	0.97	7184
2.00	0	7.10	2.93	0.83	9140	11.50	5.27	0.94	7296
2.10	1	7.08	2.91	0.79	9258	11.46	5.23	0.90	7309
Mean ± SD (n = 6)		7.11 ± 0.04	2.93 ± 0.03	0.83 ± 0.04	9159.30 ± 0.98	11.50 ± 0.05	5.27 ± 0.04	0.94 ± 0.04	7263 ± 0.95
C Amount of modifier (% v/v)									
19.50, 25.50	−1	7.16	2.97	0.87	9112	11.56	5.30	0.98	7106
20.00, 26.00	0	7.10	2.93	0.83	9140	11.50	5.27	0.94	7296
20.50, 26.50	1	7.07	2.90	0.78	9326	11.47	5.22	0.91	7305
Mean ± SD (n = 6)		7.11 ± 0.05	2.93 ± 0.04	0.83 ± 0.05	9192.7 ± 1.27	11.51 ± 0.05	5.26 ± 0.04	0.94 ± 0.04	7235.70 ± 1.55
D: Temperature of column oven (°C)									
33	−1	7.12	2.95	0.84	9110	11.53	5.29	0.97	7288
35	0	7.10	2.93	0.83	9140	11.50	5.27	0.94	7296
37	1	7.08	2.91	0.81	9192	11.46	5.25	0.93	7315
Mean ± SD (n = 6)		7.10 ± 0.02	2.93 ± 0.02	0.83 ± 0.02	9147.3 ± 0.45	11.50 ± 0.04	5.27 ± 0.02	0.95 ± 0.02	7299.7 ± 0.19
E: Columns from different manufacturers									
Kromasil		7.14	2.96	0.85	9118	11.53	5.30	0.97	7154
Finepak		7.10	2.93	0.83	9140	11.50	5.27	0.94	7296
Mean ± SD (n = 6)		7.12 ± 0.03	2.95 ± 0.02	0.84 ± 0.01	9129 ± 0.17	11.52 ± 0.02	5.29 ± 0.02	0.96 ± 0.02	7225 ± 1.39
F: Solvents of different lots									
First lot		7.10	2.93	0.83	9140	11.50	5.27	0.94	7296
Second lot		7.12	2.95	0.84	9155	11.53	5.29	0.95	7288
Mean ± SD (n = 6)		7.11 ± 0.01	2.94 ± 0.01	0.84 ± 0.01	9147.5 ± 0.12	11.52 ± 0.02	5.28 ± 0.01	0.95 ± 0.01	7292 ± 0.08

[a]Average for three concentrations, 20.0, 40.0, and 60.0 μg mL^{-1}, of each component
[b]Four factors were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0)
[c]Retention time
[d]Retention factor
[e]Tailing factor
[f]Number of theoretical plates

LOD and LOQ

For RP-HPTLC LOD and LOQ were 0.50 and 0.70 μg band^{-1}, respectively, for bacoside A_3 and 0.80 and 1.0 μg band^{-1}, respectively, for bacopaside II. For PC-SFC the LOD and LOQ were 2.50 and 4.0 μg mL^{-1}, respectively, for bacoside A_3 and 4.5 and 6.5 μg mL^{-1}, respectively, for bacopaside II.

Specificity

For densitometric RP-HPTLC peak purity for bacoside A_3 and bacopaside II was assessed by comparing spectra acquired at the peak start (S), peak apex (M), and peak end (E) of the bands. The results obtained were r(S, M) = 0.9997 and 0.9995 for bacoside A_3 and bacopaside II, respectively, and r(M, E) = 0.9998 and 0.9997 for bacoside A_3 and bacopaside II, respectively. Correlation between standard and sample spectra was also good (r = 0.9998 and 0.9997 for bacoside A_3 and bacopaside II, respectively).

In PC-SFC the average retention times, t_R, ± standard deviation, were 7.10 ± 0.25 and 11.50 ± 0.45 min for bacoside A_3 and bacopaside II, respectively (n = 6). The mean values for height equivalent to a theoretical plate and peak purity at the peak front (PF) and peak tail (PT), calculated using the 4σ method, for bacoside A_3 and bacopaside II standards were 914 and 929, 970 and 912, and 965 and 947, respectively. These values are indicative of precise results from estimation of peak purity.

Recovery

In RP-HPTLC mean recovery from capsules was 98.46 and 98.12% for bacoside A_3 and bacopaside II, respectively. For PC-SFC respective mean recovery was 99.51 and 98.64%.

The validation data obtained are summarized in Table V.

Estimation of the Compounds in Plant Extracts

In RP-HPTLC two bands at R_F 0.34 ± 0.08 and 0.42 ± 0.06 were obtained for bacoside A_3 and bacopaside II, respectively, in the densitogram obtained from the plant extract. Peaks from other components were also present but did not interfere with the analysis (Fig. 5). In PC-SFC peaks at t_R 7.10 ± 0.25 and 11.50 ± 0.45 were obtained for bacoside A_3 and bacopaside II, respectively, in the chromatogram obtained from the extract (Fig. 6).

Table V

Summary of validation data (n = 6)

Property	HPTLC–densitometry (μg spot^{-1})		PC-SFC (μg mL^{-1})	
	Bacoside A_3	Bacopaside II	Bacoside A_3	Bacopaside II
Linearity range	5.0-75.0	5.0-80.0	10.0-120.0	10.0-150.0
Correlation coefficient	0.9989 ± 1.6	0.9980 ± 1.30	0.9997 ± 1.34	0.9992 ± 1.21
Limit of detection	0.5	0.7	2.5	4.0
Limit of quantitation	0.8	1.0	4.5	6.5
Recovery (n = 6)	98.46 ± 1.52	98.12 ± 1.95	99.51 ± 1.88	98.64 ± 1.41
Precision (% RSD)				
Repeatability of application[a]	1.57	1.84	—	—
Repeatability of measurement[a]	1.34	1.61	—	—
Inter-day (n = 6)	1.73	1.97	1.86	1.92
Intra-day (n = 6)	1.31	1.64	1.58	1.77
Specificity	0.9998[b]	0.9997	986, 995[c]	991, 989

[a]Three concentrations, three replicates each
[b]Correlation of peak purity at peak start (S), peak middle (M), and peak end (E)
[c]Peak purity at peak front and at peak tail

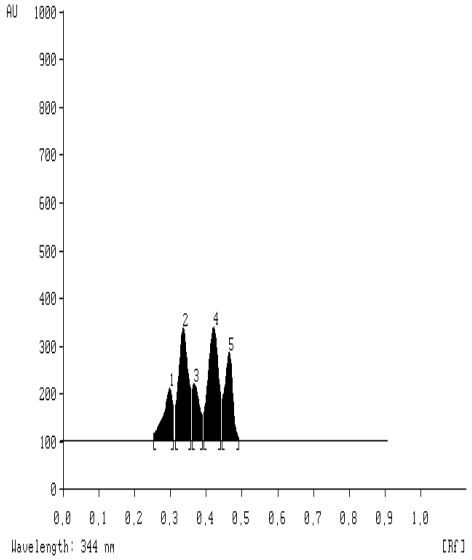

Fig. 5

Densitogram obtained from *Bacopa monnieri* extract (60.0 μg band^{-1}) scanned at 344 nm. Peaks 1, 3, and 5 are of unknown components; peaks 2 and 4 are those of bacoside A_3 and bacopaside II, respectively

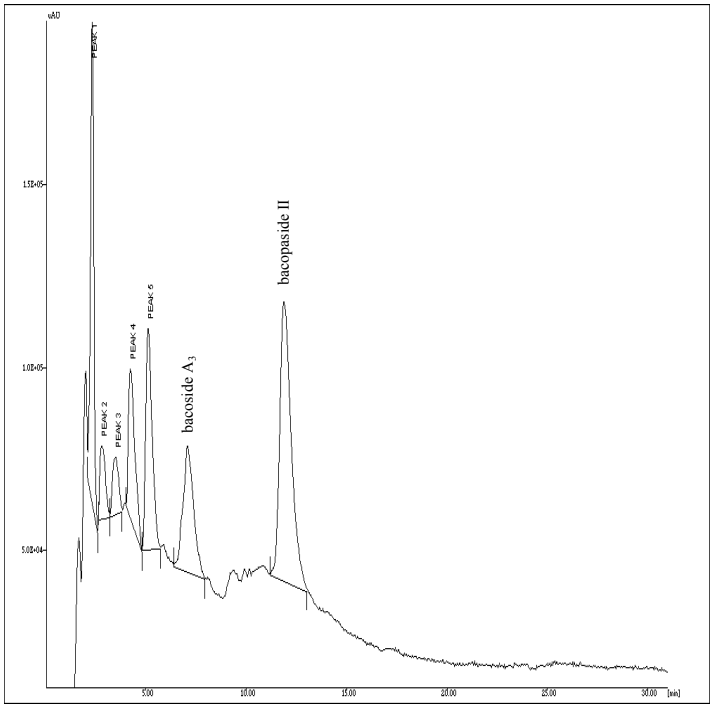

Fig. 6

Chromatogram obtained from *Bacopa monnieri* extract (100.0 μg mL^{-1}), with detection at 210 nm, showing elution of bacoside A_3 (t_R 7.12 ± 0.04) and bacopaside II (t_R 11.35 ± 0.07). Peaks 1 to 6 are of unknown components

Analysis of the Commercial Preparation

In RP-HPTLC densitometry a band at R_F 0.34 ± 0.01 for bacoside A_3 was observed in the densitogram obtained from the extract of the drug from capsule. No band was found for bacopaside II, indicating its complete absence from the marketed formulation (Fig. 7).

In PC-SFC a peak at t_R 7.12 ± 0.05 for bacoside A_3 was observed in the chromatogram and again no peak was observed for bacopaside II (Fig. 8)**.**

These results obtained for the amounts of each component in the preparation (Table VI) were less than the label claim, and the absence of bacopaside II from the formulation suggests the components have undergone degradation to some extent during processing and storage. Statistical evaluation was performed using Student's *t*-test and the *F*-ratio at 95% confidence level.

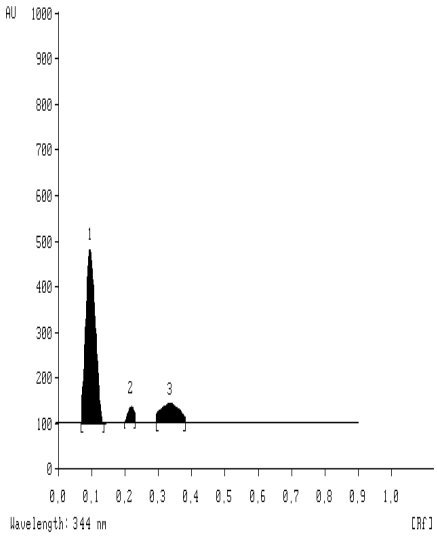

Fig. 7

Densitogram obtained at 344 nm from an extract (60.0 μg band^{-1}) of a *Bacopa monnieri* capsule. Peaks 1 and 2 are of unknown components and peak 3 is that of bacoside A_3

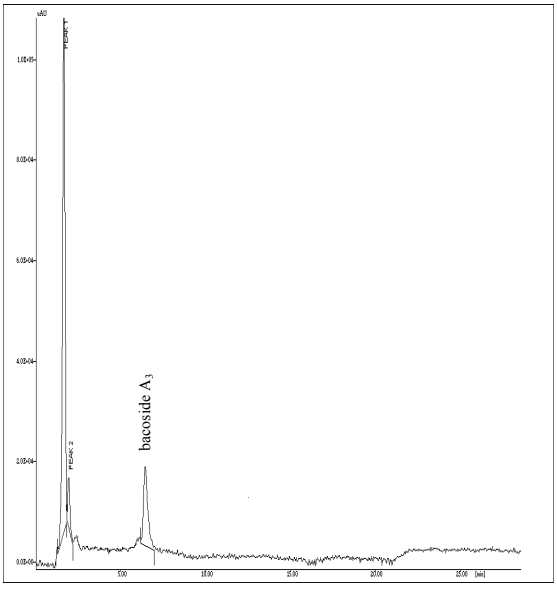

Fig. 8

Chromatogram obtained at 210 nm from an extract (100.0 μg mL^{-1}) from a *Bacopa monnieri* capsule. The chromatogram shows the presence of bacoside A_3 (t_R 7.12 ± 0.05) and the complete absence of bacopaside II. Peaks 1 to 2 are of unknown compounds

Table VI

Applicability of the methods for determination of the constituents of bacoside (n = 6)

Component	Label claim (%, *w*/*w*)	HPTLC-densitometry					PC-SFC				
		(%, *w*/*w*)[a]	RSD, %	S.E.	*t*-value[b]	*F*-value[b]	(%, *w*/*w*)[a]	RSD, %	S.E.	*t*-value[b]	*F*-value[b]
(a) Source 1	50.65										
Analysis of the herbal extract[c]											
Bacoside A_3		16.54±1.97	1.58	1.12	1.90	3.18	17.02±1.39	0.97	0.64	1.37	2.82
Bacopaside II		32.13±1.54	1.38	0.95	1.65	3.48	33.36±1.66	1.24	0.90	1.98	3.76
Total content		48.67±1.17	1.22	0.86	1.86	2.97	50.38±1.02	0.79	0.27	1.87	3.55
(b) Source 2	55.35										
Analysis of the herbal extract[c]											
Bacoside A_3		25.95±1.88	1.38	0.84	2.14	3.51	26.19±1.51	1.44	1.02	1.96	3.59
Bacopaside II		74.05±1.45	1.66	1.11	2.22	4.23	73.81±1.79	1.26	0.95	2.03	3.86
Total content		54.18±1.67	2.01	1.54	1.88	4.69	53.00±1.65	1.52	1.12	1.77	4.71
(c) Source 3	60.28										
Analysis of the herbal extract[c]											
Bacoside A_3		30.23±1.97	1.77	1.45	1.90	4.73	31.69±1.22	1.38	1.12	1.73	3.87
Bacopaside II		69.77±1.56	1.83	1.59	1.86	3.61	68.31±1.64	1.57	1.17	1.81	3.52
Total content		55.74±1.77	2.16	1.89	2.19	4.87	58.66±1.43	2.18	1.25	1.97	4.05
(d) Source 4	40.86										
Analysis of the herbal extract											
Bacoside A_3		48.14±1.74	1.41	0.68	1.62	2.98	47.57±1.46	1.32	0.51	1.66	3.57
Bacopaside II		51.86±1.52	1.26	0.40	1.89	3.16	52.43±1.81	1.53	0.89	2.05	4.12
Total content		39.37±1.63	1.35	0.57	2.17	4.14	40.45±1.64	1.37	0.70	2.23	4.66
(e) Analysis of capsule	250 mg										
Bacoside A_3		27.24±1.95	1.57	1.21	1.67	3.78	28.86±1.75	1.40	1.13	1.85	3.98
Bacopaside II		0.00					0.00				

[a]Amount found
[b]The theoretical *t*- and *F*-values are 2.57 and 5.05, respectively (P = 0.05)
[c]Standardized extract

Comparison of RP-HPTLC and PC-SFC–DAD

To test differences between the RP-HPTLC and PC-SFC methods statistical tests were performed at the level of confidence 95% (P = 0.05). The F_{stat} values (samples, column, and interaction) for bacoside A_3 and bacopaside II are shown in Table VII. The t_{stat} values are shown in Table VIII. The values of t_{stat} obtained are lower than two-tailed t_{crit} value (2.77), implying there is no significant difference between the methods.

Effect of Separation Conditions

The effect of pressure and modifier concentration on the separation of the saponins by PC-SFC was investigated by varying these conditions. The outlet pressure of the column was changed from 20 to 30 MPa with

Table VII

Two-way ANOVA test of the total bacoside content of six different samples, in duplicate, by HPTLC–densitometry and PC-SFC

Sample	HPTLC[a]		PC-SFC[a]	
	1st Sampling	2nd Sampling	1st Sampling	2nd Sampling
1	98.02	99.54	98.77	99.262
2	98.29	98.81	99.13	98.10
3	99.12	98.50	98.69	99.54
4	98.86	99.71	99.18	98.83
5	99.17	98.63	99.44	99.21
6	98.91	98.18	98.73	98.91

Anova: Two-factor with replication

Summary	HPTLC	PC-SFC	Total
Set 1			
Count	6	6	12
Sum	592.37	593.94	1186.31
Average	98.72833333	98.99	98.85916667
Variance	0.218536667	0.09284	0.160208333
Set 2			
Count	6	6	12
Sum	593.37	593.85	1187.22
Average	98.895	98.975	98.935
Variance	0.36499	0.24931	0.280972727
Total			
Count	12	12	
Sum	1185.74	1187.79	
Average	98.81166667	98.9825	
Variance	0.272815152	0.155584091	

ANOVA

Source of variation	*SS*	*df*	*MS*	*F*	*P*-value	*F*-crit
Sample	0.034504167	1	0.034504167	0.149098	0.703476	4.35125
Columns	0.175104167	1	0.175104167	0.756654	0.394698	4.35125
Interaction	0.049504167	1	0.049504167	0.213916	0.648708	4.35125
Within	4.628383333	20	0.231419167			
Total	4.887495833	23				

[a]The results are presented as percentage of declared amount of total bacoside content in extract

[b]*F*-stat < *F*-crit

Table VIII

Average results from determination of total bacoside content by HPTLC–densitometry and PC-SFC and their correlation by paired *t*-test

Sample	HPTLC[a]	PC-SFC [a]
1	98.12	98.69
2	99.69	98.78
3	98.72	99.10
4	99.08	98.56
5	98.46	99.76
6	97.93	99.15

t-Test: paired two sample for means

	98.12	98.69
Mean	98.776	99.07
Variance	0.43663	0.2069
Observations	5	5
Pearson Correlation	−0.588476902	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.658299883	
P (*T* ≤ *t*) one-tail	0.273153418	
t Critical one-tail	2.131846486	
P (*T* ≤ *t*) two-tail	0.546306837	
t Critical two-tail	2.776450856	
t-stat < *t* critical		

[a]The results are presented as percentage of declared amount of total bacoside content in extract

the composition of the mobile phase kept constant. Thermodynamic data were calculated from plots of ln k against $1/T$ and of ln α against $1/T$ (Figs 9–12). Increasing the pressure had little significant effect on the thermodynamic data calculated for the saponins, as shown in Table IX. The concentration of methanol in the mobile phase was altered from 13 to 26% (*v*/*v*), keeping the pressure constant at 25 MPa, and the temperature dependence of k and α was studied. The concentration of methanol in the mobile phase also had little effect on the thermodynamic data. Separation of bacopaside II was improved by reducing the temperature and that of bacoside A_3 was improved by increasing the temperature.

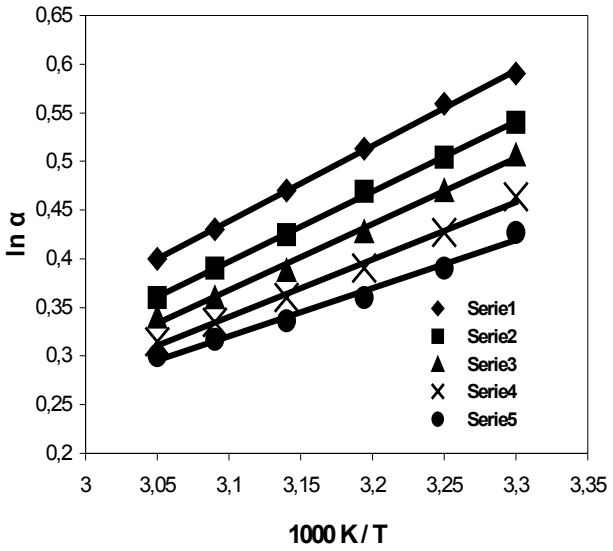

Fig. 9

Representative plot of selectivity (α) for bacoside A_3 against the reciprocal of temperature (T). The plot was used to calculate the change in molar enthalpy at pressures ranging from 20 MPa (series 1) to 30 MPa (series 5)

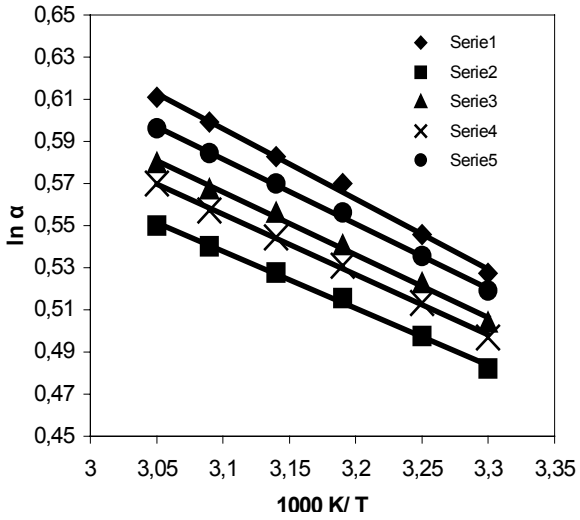

Fig. 10

Representative plot of selectivity (α) for bacopaside II against the reciprocal of temperature (T). The plot was used to calculate the change in molar enthalpy at pressures ranging from 20 MPa (series 1) to 30 MPa (series 5)

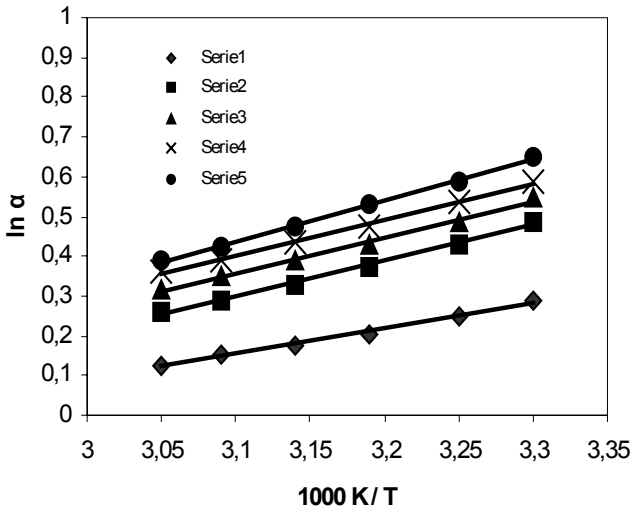

Fig. 11

Representative plot of selectivity (α) for bacoside A_3 against the reciprocal of temperature (T). The plot was used to calculate the change in molar enthalpy for modifier concentrations ranging from 13% (*v*/*v*) (series 1) to 26% (*v*/*v*) (series 5) at constant pressure (25 MPa)

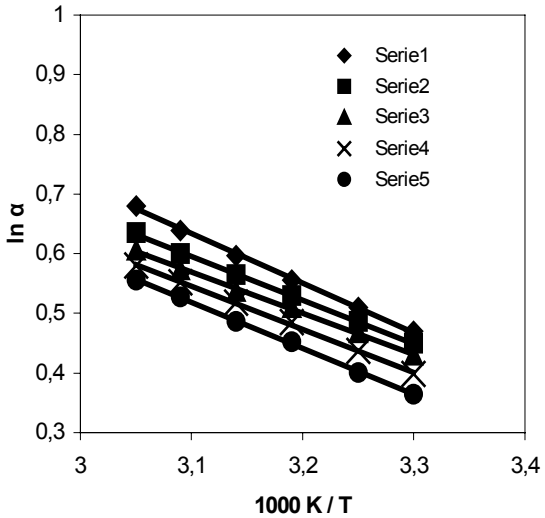

Fig. 12

Representative plot of selectivity (α) for bacopaside II against the reciprocal of temperature (T). The plot was used to calculate the change in molar enthalpy for modifier concentrations ranging from 13% (*v*/*v*) (series 1) to 26% (*v*/*v*) (series 5) at constant pressure (25 MPa)

Table IX

Effect of pressure and mobile-phase methanol content on thermodynamic data obtained by use of PC-SFC (the enthalpy units: kJ mol^{-1}; the entropy units: kJ mol^{-1} K^{-1})

	Bacoside A_3				Bacopaside II			
	$\Delta H°$	$\Delta S°$	$\Delta\Delta H°$	$\Delta\Delta S°$	$\Delta H°$	$\Delta S°$	$\Delta\Delta H°$	$\Delta\Delta S°$
Outlet pressure (MPa)								
20	13.47	28.52	6.40	16.30	8.81	8.65	+2.74	+13.55
22	14.63	33.17	5.99	15.30	12.97	23.20	+2.58	+12.72
25	11.81	25.36	5.65	14.38	13.14	24.69	+2.49	+12.39
28	10.97	23.61	4.91	12.39	11.72	20.95	+2.41	+11.97
30	9.73	20.54	4.07	10.06	9.81	15.55	+2.24	+11.39
Methanol (% *v*/*v*)								
13.04[a], 20.0[b]	16.38	34.34	5.32	15.29	22.95	50.55	+6.90	+26.69
16.67, 23.08	22.70	56.70	7.48	20.62	23.36	53.96	+6.07	+23.78
20.0, 25.93	19.45	49.05	7.50	20.29	19.70	44.98	+5.74	+22.45
23.08, 28.57	18.12	48.39	7.65	20.45	21.70	54.79	+5.98	+23.19
25.93, 31.03	16.30	45.89	8.65	23.28	25.11	69.00	+6.40	+24.11

We were interested in RP-HPTLC and PC-SFC as straightforward and rapid means of improving the separation of marker compounds, and the maximum number of other, unknown, components, to enable identification of the herbal drugs and to assess drug quality. Because RP-HPTLC separation relies on Kubelka–Munk theory compounds absorbing at short UV wavelengths can easily be separated and quantified with high sensitivity because organic solvents are removed from the TLC plate during drying. The excellent transparency of supercritical carbon dioxide at short UV wavelengths prompted us to investigate the feasibility of PC-SFC for standardization of Bacopa extract. The coupling of SFC with photodiode-array detection is also a very promising technique for standardization of herbal drugs the components of which have different λ_{max} values, because this has a direct effect on the sensitivity of the technique. Quality-control laboratories could use these chromatographic techniques to authenticate and identify herbal medicines.

CONCLUSION

PC-SFC enables resolution of compounds that were partially separated by liquid chromatography, demonstrating the utility of using complimentary chromatographic techniques. In contrast with allopathy, herbal raw

materials are processed during manufacture to convert them into better-absorbed and more efficacious products. For this reason a raw material may not be detected in the finished herbal product. RP-HPTLC is, perhaps, a practical solution to characterization of complex herbal formulations.

The extreme difficulty of standardizing multi-component herbal formulations means that great emphasis on quality control of raw material is absolutely essential. RP-HPTLC is very useful for obtaining good resolution of the main components present in a crude drug extract. Use of PC-SFC with photodiode-array detection is useful in herbal analysis when it is important to generate high-quality information in limited time and with limited resources, and when chromatographic profiles enable fundamental assessment of the suitability of separation of markers and the maximum number of unknown components. Improving PC-SFC separations affords the opportunity to assess the accuracy of the profiles generated with good resolution between the markers and other, unknown, components. This technique could be exploited further for such applications. The temperature dependence of 'k' and 'α' for the optical isomers of bacoside by PC-SFC was studied. In the temperature range examined separation of bacoside A_3 is enthalpically controlled whereas separation of bacopaside II was entropy controlled.

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