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Liquid chromatographic method for analysis of saponins in *Maesa balansae* extract active against leishmaniasis

Stefanie Leonard[a], Raul Capote[a], Nils Germonprez[b], Luc Van Puyvelde[b], Norbert De Kimpe[c], Hans Vermeersch[b], Jan Rosier[b], Louis Maes[b], Eugène Roets[a], Jos Hoogmartens[a,*]

[a]*Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, E. Van Evenstraat 4, B-3000 Leuven, Belgium*
[b]*Tibotec N.V., B-2800 Mechelen, Belgium*
[c]*Vakgroep Organische Chemie, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Coupure Links 653, B-9000 Ghent, Belgium*

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Abstract

A liquid chromatographic method was developed for the separation of six related triterpenoid saponins in *Maesa balansae* extracts with different purity, active against leishmaniasis. As stationary phase a Hypersil BDS C_{18} column (3 μm), 100×4.6 mm was used. The mobile phase was a mixture of methanol, acetonitrile, 5% (m/v) ammonium acetate, pH 6.5 and water. A linear gradient was developed for the analysis of crude extracts. An isocratic method was developed to analyze purified samples that mainly contained saponins 3 and 4, the most active saponins. The isocratic LC method was optimized and the robustness was evaluated with an experimental design. The method showed good selectivity, repeatability, linearity and sensitivity.

Keywords: *Maesa balansae*; Plant materials; Saponins; Terpenoids

1. Introduction

Leishmaniasis is an infectious disease in man and animal caused by protozoa of the genus *Leishmania* [1,2]. It is transmitted to man most often by the bites of infected female phlebotamine sandflies. Human leishmaniasis can be classified into a cutaneous, mucocutaneous and visceral form.

Extracts from leaves of the *Maesa balansae* plant appeared to have a specific activity against *Leishmania*. The antileishmanial activity of these extracts was attributed to six related triterpenoid saponins. Those saponins were isolated from a crude saponin mixture obtained after extraction of the leaves of *Maesa balansae* with methanol–water (9:1), followed by liquid–liquid partitioning between *n*-butanol–water, suspension in acetone and final puri-

*Corresponding author. Tel.: +32-16-323-442; fax: +32-16-323-448.
E-mail address: jos.hoogmartens@farm.kuleuven.ac.be (J. Hoogmartens).

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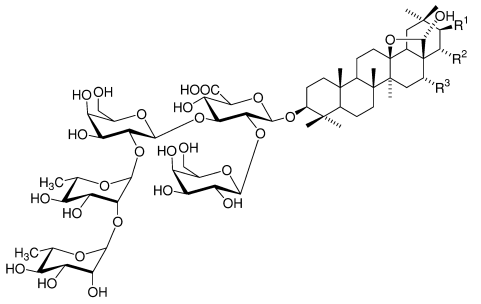
fication by preparative liquid chromatography (LC). Each step was guided by results of in vitro anti-leishmanial tests [3].

Saponins are in general plant secondary metabolites with a wide range of biological activities [4,5]. Most of these activities are due to their characteristic surface activity. The structures of these six saponins of interest were determined by Tibotec (Mechelen, Belgium) and are shown in Fig. 1.

In this paper a gradient elution LC method is described, utilizing a base-deactivated silica (BDS) reversed phase, which allows separation of the six major saponins. The method development was performed on crude extracts, which contained the six saponins of interest. Later, the method was adapted for the isocratic analysis of more purified samples, which mainly contained the two most active components, i.e., saponins 3 and 4.



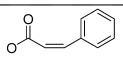
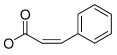
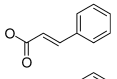
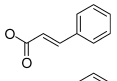
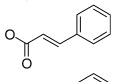
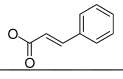
Saponin	R^1	R^2	R^3
1			OH
2			OH
3			OH
4			OH
5			OAc
6			OAc

Fig. 1. Structures of the six saponins of interest in *Maesa balansae* extracts.

2. Experimental

2.1. Reagents

Acetic acid and anhydrous ammonium acetate were obtained from Acros Organics (Geel, Belgium), sodium hydroxide pellets and methanol from BDH (Poole, UK), acetonitrile HPLC-R grade from Biosolve (Valkenswaard, The Netherlands). Water was distilled twice before use. Mobile phases were degassed by purging with helium gas for 1 min before use.

2.2. Apparatus

LC analyses were performed using a L-6200 Intelligent pump (Merck–Hitachi, Darmstadt, Germany), equipped with a TSP Spectra Series AS 100 autosampler set to inject 20 μl (San Jose, CA, USA), a Merck–Hitachi L-4200 UV–Vis detector set at 275 nm and a HP 3396 Series III integrator (Agilent Technologies, Avondale, PA, USA). The Hypersil BDS C_{18} column 100×4.6 mm I.D., 3 μm (Shandon, Runcorn, UK) was immersed in a water bath, heated by a Julabo EM thermostat (Julabo, Seelbach, Germany). A flow-rate of 1.0 ml/min was used. The pH measurements were performed on a Consort C831 Multichannel analyzer (Turnhout, Belgium).

2.3. Reference substances and samples

Samples of *Maesa balansae* extract and reference substances of each of the six saponins of interest were obtained from Tibotec. The structures of all the reference substances were confirmed by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

2.4. Software

Experimental design, optimization and robustness testing were supported by Modde 4.0 software (Umetri, Umeå, Sweden).

3. Results and discussion

3.1. Gradient method for the analysis of crude samples

Preliminary work using an isocratic elution on C_{18} reversed-phase material with a mobile phase of acetonitrile, methanol, ammonium acetate and water gave reasonably good chromatographic separation between the six saponins of interest. The use of only one organic modifier in the mobile phase was studied with methanol, acetonitrile, tetrahydrofuran or 2-propanol. In all cases the separation between peaks was poor. A methanol–acetonitrile mixture in the mobile phase was needed to obtain sufficient resolution. The influence of the concentration (ratio aqueous–organic phase) of a 5% (m/v) ammonium acetate solution in the mobile phase was examined. The results showed that at least 40% (v/v) of this 5% (m/v) solution was required to achieve improved peak shape and resolution. Isocratic elution was found to be insufficient for the apolar components in the extracts to be eluted. Therefore, a gradient elution method was developed, with as mobile phases: (A) 5% (m/v) NH_4OAc, pH 6.5–methanol–acetonitrile–water (40:16:27:17, v/v) and (B) 5% (m/v) NH_4OAc, pH 6.5–methanol–acetonitrile–water (40:16:40:4, v/v). A three-step gradient was introduced: first isocratic elution with mobile phase A (30 min), then a linear gradient to A–B (50:50) (30–60 min) and as third step a linear gradient to 100% B (60–70 min). The latter step was necessary for the elution of the most apolar components in the samples. The influence of the concentration of organic modifier in function of gradient elution time, the pH and the temperature was investigated and evaluated by measuring the resolution (Table 1). This parameter was calculated according to the European Pharmacopoeia [6]. To study the gradient, the final concentration in the second step (30–60

Table 1
Influence of gradient, pH and temperature on resolutions between saponin 1 and saponin 2 (R_{S1-S2}), saponin 3 and saponin 4 (R_{S3-S4}) and saponin 5 and saponin 6 (R_{S5-S6})

		Resolution		
		R_{S1-S2}	R_{S3-S4}	R_{S5-S6}
Gradient[a,b]	0–30 min: 100% A 30–60 min: A–B (30:70) 60–70 min: 100% B	1.97	2.43	1.25
	0–30 min: 100% A 30–60 min: A–B (35:65) 60–70 min: 100% B	2.05	2.49	1.33
	0–30 min: 100% A 30–60 min: A–B (50:50) 60–70 min: 100% B	2.11	2.48	1.63
pH[b,c]	6	1.56	1.67	1.08
	6.5	2.11	2.48	1.63
	7	1.76	1.89	1.12
Temperature (°C)[a,c]	35	1.76	2.20	1.18
	45	2.17	2.44	1.47
	55	2.29	2.50	1.90

Column, Hypersil BDS C_{18}, 3 μm, 100×4.6 mm; mobile phase, 5% (m/v) NH_4OAc, pH *x*–MeOH–acetonitrile–water; (A) 40:16:27:17 (v/v); (B) 40:16:40:4 (v/v).

[a] pH 6.5.

[b] Column temperature, 40 °C.

[c] Gradient program, 0–30 min (100% A), 30–60 min (A–B, 50:50), 60–70 min (100% B).

min) was changed (A–B, 30:70 or 35:65 or 50:50). The latter gave the best peak resolution. Three different pH values were evaluated (6, 6.5, 7) and the best results were obtained with a pH of 6.5. During the evaluation of the influence of the temperature (35 to 55 °C), it was observed that an increase in the temperature resulted in better peak resolution and that an unknown impurity coeluted with saponin 6 below a temperature of 45 °C. On the other hand, it is preferable to work with the lowest temperature as possible for reasons of stability. Therefore, it was decided to adapt the gradient program for further improvement of the separation between saponins 5 and 6 and the unknown impurity at a temperature of 45 °C. The isocratic elution with mobile phase A was extended to 40 min, the second step (40–60 min) was a linear gradient to 100% B and the last step was isocratic elution with mobile phase B (60–70 min). Fig. 2 shows a typical chromatogram of a *Maesa balansae* extract containing the six saponins.

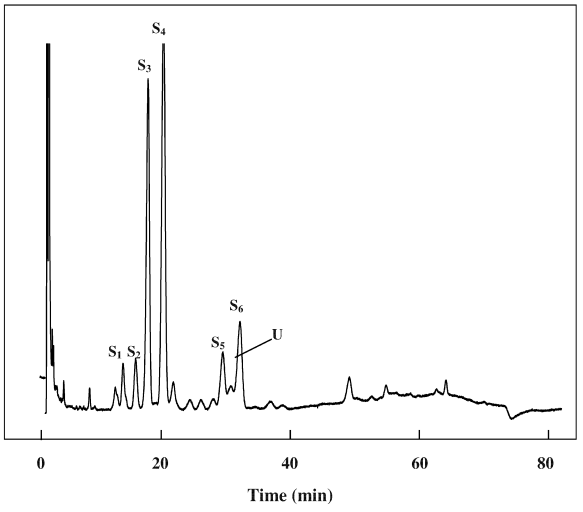


Fig. 2. Typical chromatogram of a *Maesa balansae* extract containing the six saponins (S_1–S_6). Column, Hypersil BDS C_{18}, 3 μm, 100×4.6 mm, maintained at 45 °C; mobile phase, 5% (m/v) NH_4OAc, pH 6.5–methanol–acetonitrile–water, (A) (40:16:27:17, v/v); (B) (40:16:40:4, v/v); gradient program, 0–40 min (100% A), 40–60 min (linear to 100% B), 60–70 min (100% B); flow–rate, 1.0 ml/min; sample concentration, 0.4 mg/ml; injection volume, 20 μl; UV detection at 275 nm. Peaks: S_1–S_6 = saponins 1–6, U = unknown impurity.

3.2. Isocratic method for purified samples mainly containing saponins 3 and 4

The most active components in the *Maesa balansae* extracts were found to be saponins 3 and 4. Therefore, it was necessary to have available an analytical method for preparations, containing mainly saponins 3 and 4. In these preparations, saponins 1 and 2 were only present in small amounts and saponins 5 and 6 were absent. Development was focused on separation of impurities from saponins 3 and 4, with an isocratic method if possible.

Because of the fact that saponins 5 and 6 were not present in the samples, it was not necessary to have a temperature of 45 °C or higher. A temperature of 40 °C gave the best separation of peaks 3 and 4 and their impurities. The influence of the mobile phase components was investigated in a preliminary study. The influence of the pH was not significant. The concentration of ammonium acetate only slightly influenced the separation between saponin 2 and an unknown impurity. As before it was observed that both acetonitrile and methanol were needed in the mobile phase. The ratio acetonitrile–methanol was important.

3.3. Optimization of the isocratic method

Optimization was performed by using an experimental design and multivariate analysis with Modde 4.0 software. Three factors, which appeared from previous work to have influence on the separation of the saponins and their impurities, were optimized by a central response surface modeling (RSM) experiment [7]. These factors and the ranges studied are summarized in Table 2A. The mobile phase was a mixture of 5% (m/v) NH_4OAc, pH 6.5, methanol, acetonitrile and water. When the acetonitrile–methanol ratio was changed, the total solvent strength was kept constant, so that the retention times were similar. Three response variables were examined in the experimental design, i.e., the resolution between saponins 3 and 4 (R_{S3-S4}), the selectivity between unknown impurity 1 and saponin 3 (S_{U1-S3}) and the number of impurities separated from saponins 3 and 4 (N_{imp}). These were the most critical parameters in the separation. The resolution

Table 2
Factorial analysis nominal values in (A) the optimization of the isocratic method and (B) the robustness test, corresponding to −1, 0 and +1 levels

	A			B		
	(−1)	(0)	(+1)	(−1)	(0)	(+1)
Temperature (°C)	35	40	45	42.5	45	47.5
Concentration (%, v/v) of 5% (m/v) ammonium acetate in mobile phase	30	35	40	37.5	40	42.5
Acetonitrile–methanol ratio	1	1.5	2	1.8	2	2.2

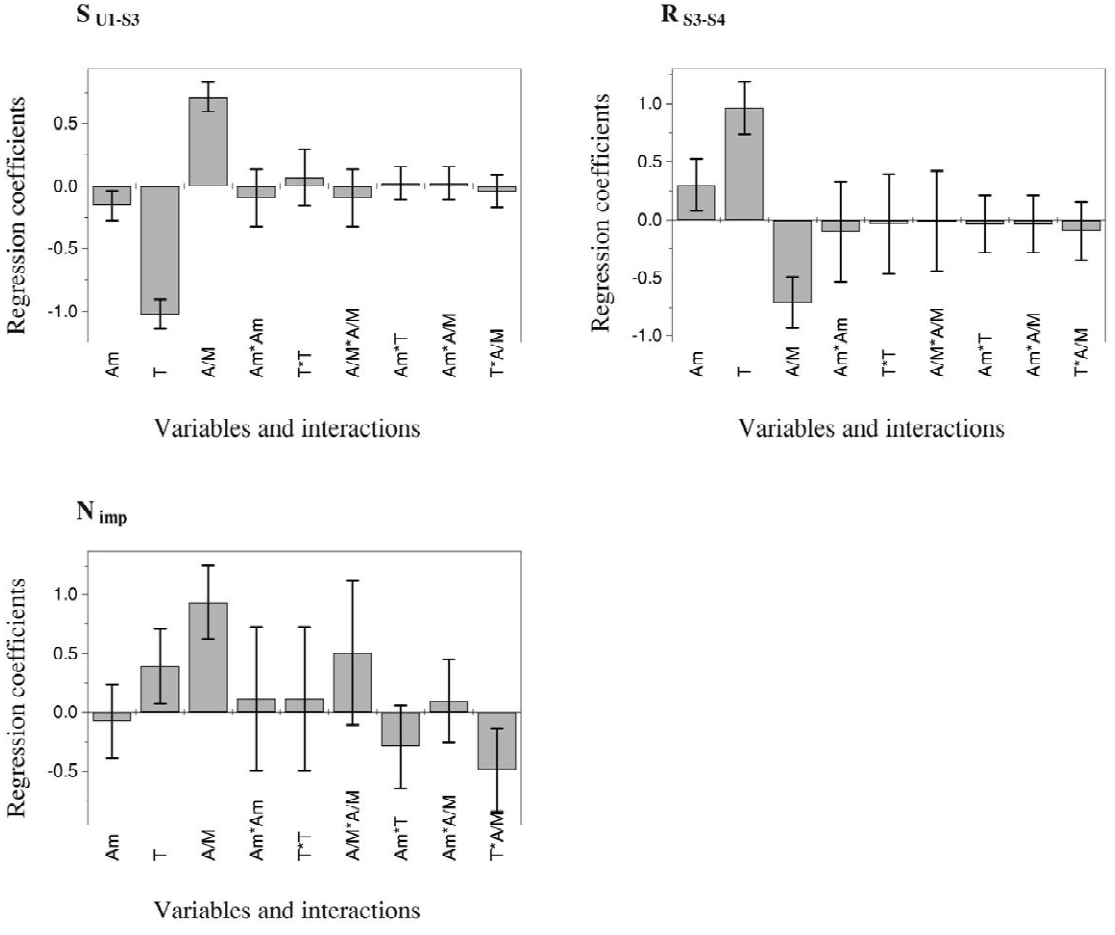


Fig. 3. Regression coefficients plots for the response variables, for the evaluation of the optimization of the isocratic method. S_{U1-S3} = selectivity between unknown impurity 1 and saponin 3, R_{S3-S4} = resolution between saponins 3 and 4, N_{imp} = number of impurities separated from saponins 3 and 4 [Am = 5% (m/v) ammonium acetate concentration in mobile phase (%, v/v), T = temperature (°C), A/M = acetonitrile–methanol ratio].

and the selectivity were calculated according to the European Pharmacopoeia [6].

A central composite design was used for the purpose of this study. The experiment needed 17 experiments in total ($2^k + 2k + 3$, with k = number of variables) including three center points. In particular, this model consists of ($2^k + 3$) points of a full factorial design and of $2k$ star points to enable this model to estimate the response curvature plot. The star points are located at the center and both extreme levels of the experimental domain [7,8].

The statistical relationship between a response Y and the variables X_i, X_j, ... can be described by Taylor expansion:

$$Y = \beta_0 + \chi\beta_i X_i + \chi\beta_{ij} X_i X_j + \chi\beta_{ii} X_i^2 + E$$

where β = regression coefficient and E = overall experimental error [7].

The linear coefficient for the experimental variables, β_i describes their quantitative effect in the model. The cross product, β_{ii} measures the interaction effect between the variables and the square term $\beta_{ii} X_i^2$ describes the non-linear effect on the response [6]. The 95% confidence interval was expressed in terms of error bar over the coefficient. If the coefficient is smaller than this interval, the variation of the response caused by changing the variable is smaller than the experimental error. This means that the variable is considered not to be significant.

Fig. 3 illustrates that the temperature has a significant effect both on S_{U1-S3} and R_{S3-S4}. The effect is negative for the former and positive for the latter. The temperature has little influence on N_{imp}. A temperature of 45 °C was chosen because of the better separation between the main components and the shorter analysis time. In the concentration range investigated, the ammonium acetate concentration has no significant influence. It was kept at 40% (v/v) as in the gradient elution. An increase in the acetonitrile/methanol ratio increases S_{U1-S3} and N_{imp} and decreases R_{S3-S4}. This corresponds with an earlier observation that acetonitrile increases the separations between the saponins and their impurities while methanol improves the separation between saponins 1 and 2 and saponins 3 and 4.

In order to find the best compromise between the three responses and the analysis time, an ammonium acetate concentration of 40% (v/v), an acetonitrile–methanol ratio of 2 and a temperature of 45 °C were selected. A typical chromatogram using the optimal conditions is shown in Fig. 4.

3.4. Robustness

Robustness is an important feature of analytical method development. It evaluates the influence of small changes in the operating conditions of the analytical procedure on measured or calculated responses. Appropriate experimental design can also be used for this purpose [9]. The experimental design described above in the optimization, could not be used for this purpose because the central value of the factors in this design did not correspond with the optimal condition. A new central composite design was made with the optimal conditions of the factors as central value (Table 2B). The same response variables were used, i.e., R_{S3-S4}, S_{U1-S3} and N_{imp}. Fig. 5 shows that in the temperature range from 44 to 46 °C and for an acetonitrile–methanol ratio from 1.9 to 2.1, the method is robust.

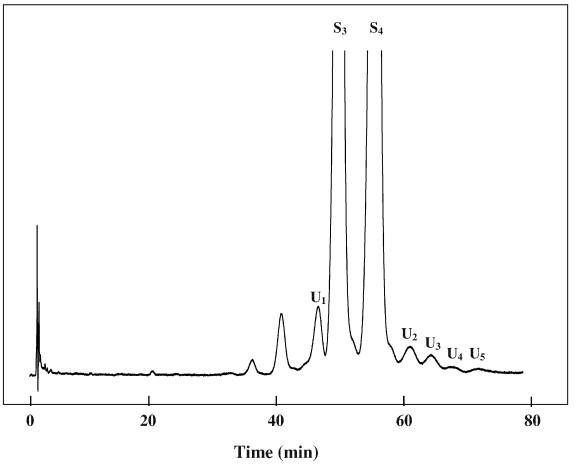


Fig. 4. Typical chromatogram of a *Maesa balansae* extract mainly containing the most active saponins 3 and 4. Column, Hypersil BDS C_{18}, 3 μm, 100×4.6 mm, maintained at 45 °C; mobile phase, 5% (m/v) NH_4OAc, pH 6.5–methanol–acetonitrile–water (40:12.85:25.7:21.45, v/v); flow-rate, 1.0 ml/min; sample concentration, 0.56 mg/ml; injection volume, 20 μl; UV detection at 275 nm. Peaks: S_3–S_4 = saponins 3–4, U_1–U_5 = unknown impurities 1–5.

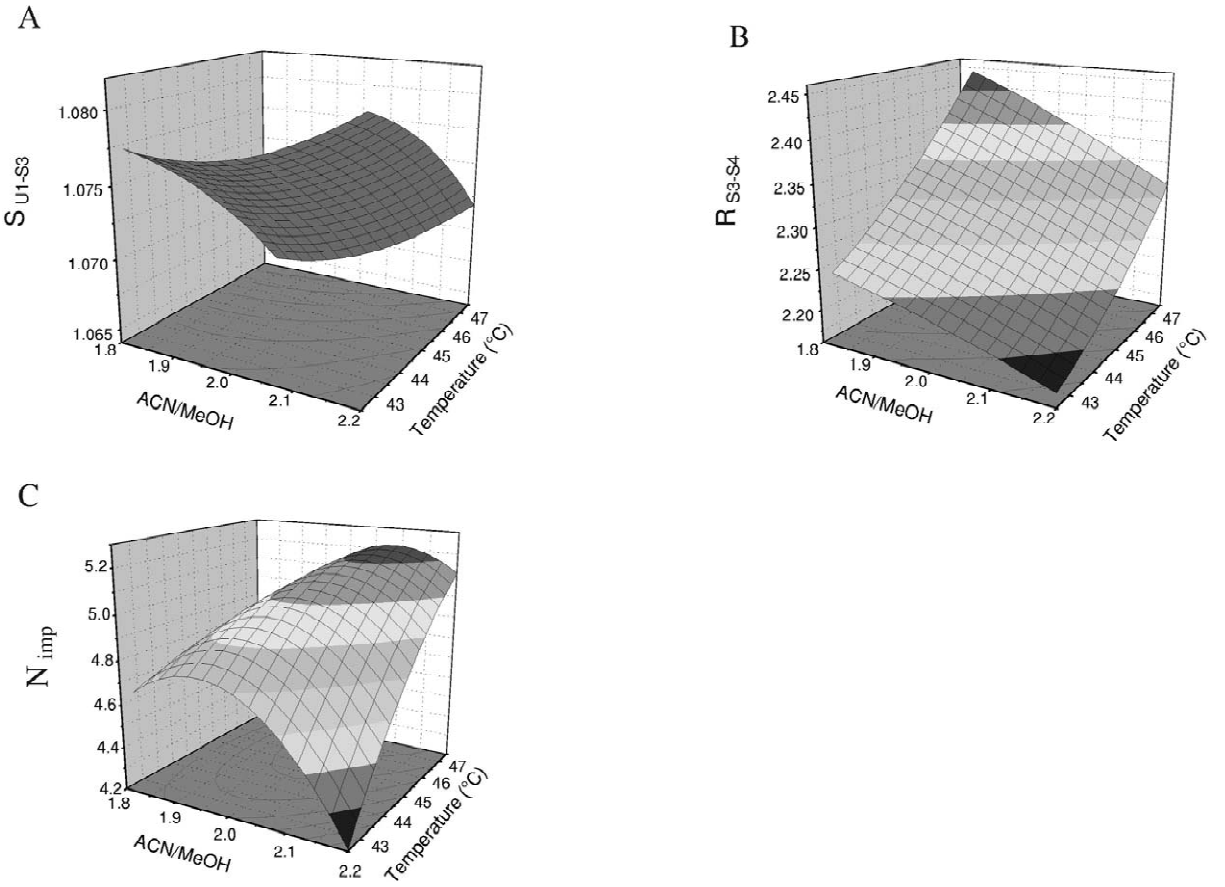


Fig. 5. Response surface plots of the response variables as a function of significant separation parameters, for the evaluation of the robustness. (A) S_{U1-S3} = selectivity between unknown impurity 1 and saponin 3, (B) R_{S3-S4} = resolution between saponins 3 and 4, (C) N_{imp} = number of impurities separated from saponins 3 and 4. The 5% (m/v) ammonium acetate concentration in the mobile phase was kept at 40% (v/v).

3.5. Quantitative aspects

Quantitative aspects of this analytical method were investigated with a purified sample mainly containing saponins 3 and 4. Repeatability, linearity, limit of quantification (LOQ) and limit of detection (LOD) were calculated for saponins 3 and 4. The repeatability of the method was performed with a 0.6 mg/ml solution. The relative standard deviations (RSDs) of the peak area of saponins 3 and 4 were 0.2 and 0.7%, respectively ($n=6$). The inter-day repeatability was performed during 6 days ($n=3$). For saponins 3 and 4 the RSDs on all the results were 2.5 and 3.7%, respectively. The calibration curves obtained by replicate analysis ($n=3$) of a series of analyte concentrations corresponding to 1, 5, 25, 50, 75, 100, 120 and 150% calculated versus a 0.6 mg/ml solution were subjected to linear regression analysis. The results are shown in Table 3. The results for the LOQ (signal-to-noise ratio $S/N=10$) and the LOD ($S/N=3$) are calculated versus a 0.6 mg/ml solution and are summarized in Table 4. These parameters were the same for both saponins 3 and 4.

4. Conclusion

The Hypersil BDS C_{18} (3 μm) stationary phase shows good selectivity towards the six saponins. For

Table 3
Linearity[a] of saponins 3 and 4

	Saponin 3	Saponin 4
Regression equation	$y = 996\,758x + 80\,508$	$y = 1\,261\,767x + 42\,436$
Correlation coefficient, r	0.9997	0.9997
Standard error of estimate, $S_{y,x}$	161 887	213 774
Number of concentrations studied, n_c	8	8
Number of injections/concentration, n_i	3	3

[a] Linearity range from 1 to 150% relative to 0.6 mg/ml sample. y = Peak area, x = mass (μg) of sample injected. Saponins 3 and 4 are present in this sample in about equal amounts.

Table 4
Limit of quantification (LOQ) and limit of detection (LOD) for saponins 3 and 4

		Saponin 3	Saponin 4
LOQ[a]	Relative concentration (%)[b]	0.60	0.60
$S/N = 10$	Absolute mass (ng)[c]	36	36
	RSD on areas (%) ($n = 6$)	8	7
LOD[a]	Relative concentration (%)[b]	0.22	0.22
$S/N = 3$	Absolute mass (ng)[c]	13	13

[a] S/N = Signal-to-noise ratio.
[b] Relative to 0.6 mg/ml.
[c] Injected volume = 20 μl.

the analysis of crude extracts containing the six saponins, a gradient elution method was necessary to elute all the components. For the analysis of purified samples, which mainly contain saponins 3 and 4, an isocratic method is sufficient for the separation from the minor components.

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