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ORIGINAL ARTICLE

Determination of Coumarins from *Pterocaulon balansae* by an Ultra-Fast Liquid Chromatography Method in Topical Applications

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Abstract

Coumarins from *Pterocaulon balansae* Chodat, Asteraceae, have received increasing attention due to their biological activities, especially for the treatment of skin disorders. This study aimed to develop and validate high-performance and ultra-fast liquid chromatography methods for the analytical determination of coumarins in extracts, in topical formulations, as well as in porcine skin after *in vitro* permeation/retention studies. The chromatographic conditions consisted of a mobile phase with 0.1% formic acid (A) and acetonitrile (B) using a gradient elution; the flow rate and a column oven temperature: HPLC-UV, 1 ml min^{-1}; 30 °C and UFLC-DAD, 0.55 ml min^{-1}; 55 °C for 45 min and 8 min, respectively. The chemical marker 5-methoxy-6,7-methylenedioxycoumarin was used for the construction of the linearity curve in a concentration range from 0.1 to 7.5 μg ml^{-1}. A low matrix effect was observed. The method robustness was confirmed using the Plackett-Burman experimental design. These methodologies proved to be linear, precise, and accurate for the determination of seven coumarins in analytical and bioanalytical samples in both approaches. However, UFLC analysis time was shortened to about one-fourth the time and consumes significantly less eluent, being more eco-friendly in comparison with HPLC.

Keywords Coumarin assay · Permeation/retention · Porcine skin layers · Topical nanoemulsions · UFLC/HPLC

Introduction

Members of the genus *Pterocaulon* Ell., Asteraceae, have been employed in traditional medicine for the treatment of several diseases. Different activities of *Pterocaulon* extracts (*P. balansae* Chodat, *P. polystachyum* DC., and *P. alopecuroides* (Lam.) DC.) for the treatment of skin disorders have been described (Goleniowski et al. 2006), especially against fungal infections (Stein et al. 2005, 2006; Stopiglia et al. 2011), which demonstrated a pronounced activity against the dermatophytes *Trichophyton rubrum*, *T. mentagrophytes*, and *Microsporum gypseum* (Stein et al. 2006), as well as, on clinical isolates of the fungus *Sporothrix schenckii* (Stopiglia et al. 2011). These biological activities have been associated to their coumarin contents (Medeiros-Neves et al. 2018). Among them, 5-methoxy-6,7-methylenedioxycoumarin (**1**) occurs in several species and has exhibited cytotoxicity (Riveiro et al. 2004; Vianna et al. 2012) and insecticidal (Vera et al. 2006) potential. Our research group has previously investigated the qualitative and quantitative coumarin composition of *P. balansae* aqueous, hexane, and supercritical fluid extracts (Medeiros-Neves et al. 2015; Panatieri et al. 2017; Torres et al. 2017), which led us to determine the presence of seven coumarins. However, the quantification of coumarins in crude extracts proved to be a difficult task. In fact, the procedure requires a long period of analysis (almost 40 min) to achieve the separation of these coumarins with a satisfactory resolution. Different chromatographic techniques can be used to improve the analysis of the active compounds in products and/or biological samples (Silva et al. 2011; Högner et al. 2013; Nemitz et al. 2015; Li et al. 2016). In addition, ultra-efficient methods may enhance liquid chromatography techniques due to the application of high pressure, facilitating the

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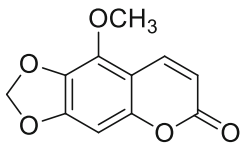
use of chromatographic columns with a reduced internal diameter, increasing peak resolution and reducing both the chromatographic run time and the mobile phase consumption (Gaikwad et al. 2010; Bucar et al. 2013; Fekete et al. 2014).

Therefore, the first aim of this study was to develop an ultra-fast liquid chromatography (UFLC) method to determine the main coumarins of *P. balansae* aqueous extract. Initial UFLC chromatographic conditions were set by using the Method Transfer Program (Prominence, Shimadzu Corporation) based on our previous report using HPLC (Medeiros-Neves et al. 2015). After that, both HPLC and UFLC methods were validated and compared to estimate coumarin contents in topical nanoemulsions and porcine ear skin samples from *in vitro* permeation/retention studies.



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Materials and Methods

Plant Material

Aerial parts of *Pterocaulon balansae* Chodat, Asteraceae, were collected in Canoas, Rio Grande do Sul, Brazil, in February 2013. The species was identified by Sérgio Augusto de Loreto Bordignon (Centro Universitário La Salle, Brazil). Voucher specimen was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN #157762). Plant collection was authorized by the Ministry of the Environment (Ministério do Meio Ambiente, MMA), Chico Mendes Institute for Biodiversity Conservation (ICMBio; SISBIO number #38017-1).

Instrumental Conditions

HPLC Analysis

The method was performed on a Shimadzu LC-20AT system (Kyoto, Japan), equipped with an UV detector (327 nm) controlled by LC-solution Multi-PDA software (Kyoto, Japan). A Phenomenex-C_{18} Synergi column (150 mm × 4.6 mm, 4 μm) coupled to a refillable pre-column filled with C_{18} reversed-phase silica was used in the analysis. The mobile phase consisted of a gradient of (A) formic acid 0.1% and (B) acetonitrile, filtered and degassed, as follows: 0–17% B (0–0.01 min), 17–20% B (0.01–10 min), 20% B (10–15 min), 20–25% B (15–20 min), 25–27% B (20–22 min), 27–30% B (22–25 min), 30–35% B (25–30 min), 35% B (30–35 min), 35–17% B (35–40 min). The system operated at flow rate of 1 ml min^{-1} for 45 min at 30 °C with the injection volume of 20 μl.

UFLC Analysis

A Shimadzu Prominence UFLC system (Shimadzu, Japan) equipped with a diode array detector (SPD-M20A) was used (327 nm). The output signal was monitored and processed using Shimadzu LC-solution Multi-PDA software (Kyoto, Japan). Chromatographic separation was performed on a Shim-pack XR C_{18} column (100 × 2.0 mm, 2.2 μm), guarded by an in-line pre-column C_{18} SecurityGuard ULTRA (Phenomenex, USA). The mobile phase consisted of gradient elution of (A) formic acid 0.1% (v/v) and (B) acetonitrile: 0–17% B (0–0.09 min), 17–25% B (0.09–1.45 min), 25% B (1.45–2.20 min), 25–32% B (2.20–2.90 min), 32–37% B (2.90–4.77 min), 37–39% B (4.77–4.90 min), 39–40% B (4.90–5.60 min), 40% B (5.60–7.20 min), 40–22% B (7.20–7.80 min), 22–17% B (7.80–8.00 min). The column was re-equilibrated with 17% B for 2 min before the next analysis. The flow rate was set in 0.55 ml min^{-1} up to 8 min at 55 °C. The injection volume was 5 μl.

Solutions

Reference solutions of compound 1 (0.1, 0.5, 1, 2.5, 5, 7.5 μg ml^{-1}) were prepared with CH_3CN:H_2O (1:1 v/v). The aqueous extract was prepared with the dried aerial parts and water 2% (w/v) at 60 °C in a stirrer with water bath (Dist-DI920) for approximately 4 h. Nanoemulsions composed of medium-chain triacylglycerides 16% (w/w), egg lecithin 4% (w/w), polysorbate 80 1% (w/w), and water up to 100% were prepared through spontaneous emulsification. Adequate aliquots of the samples in aqueous extracts and nanoemulsions were diluted in CH_3CN:H_2O (1:1 v/v), filtered, and analyzed by HPLC and UFLC.

Skin Preparations

For preparation of porcine skin layers, the skin was removed from the back of the porcine ear. After removal of subcutaneous fat and hair, the skin was cut into round pieces, and the separation of the stratum corneum was carried out through tape stripping. The first stripped tape was discarded, while the following 14 tapes were placed in test tubes and used for the stratum corneum analysis. The remaining layer (epidermis/dermis) was reduced to tiny pieces and placed in different test tubes (Meira et al. 2020). To extract the coumarins from skin layers, 2 ml of methanol was added, and the samples were kept in an ultrasound bath for 45 min, resulting in porcine epidermis/dermis skin layers. The receptor fluid used in permeation/retention studies was a mixture of 40% ethanol

in phosphate buffer pH 7.4. An adequate aliquot was filtered and analyzed in HPLC and UFLC.

Validation

The developed HPLC and UFLC methods were validated according to the official guidelines (ICH 2005; EMA 2012; FDA 2018). The results were analyzed by Student's *t* test and analysis of variance (ANOVA) using a significance level of $\alpha = 0.05$. The interference of the matrix composition was determined by injecting samples containing only matrices, and matrices spiked with the aqueous extract and the standard solution of **1** at a concentration of 5 μg ml^{-1}. The coumarins present in different matrices were identified based on their UV spectra between 200 and 400 nm and their retention times, confirmed by the Mass, UV, and NMR data as previously described (Medeiros-Neves et al. 2015). Concentrations of 0.1, 0.5, 1, 2.5, 5, and 7.5 μg ml^{-1} of standard sample (**1**) were prepared in CH_3CN:H_2O (1:1 v/v). The standard linearity curve was constructed using peak area versus the known concentrations of **1**. The linear regression line was used to determine the linearity and concentration of the six sets of samples. The detection and quantification limits (LOD and LOQ, respectively) were calculated from the linearity curve, using the values of standard deviation of the intercept (σ) and of the slope (S) (LOD 3.3 σ/S and LOQ 10 σ/S). To determine the accuracy, the aqueous extract was prepared and spiked with known amounts of analyte, at low, medium, and high concentrations of a solution for 1 (0.5, 2.5, and 5 μg ml^{-1}), performing five determinations for each concentration. The results represent the mean recovery (%) for three independent samples. The repeatability of the method was determined by analysis of five determinations of **1** in three points of the analytical curves, during the same day under the same experimental conditions. The intermediate precision values were obtained by assaying freshly prepared solutions for the construction of the analytical curve on three different days. The results were expressed in relative standard deviation (RSD %). The robustness in each matrix was investigated by the Plackett-Burman design. The factors, column oven temperature, flow rate, initial organic composition, and formic acid concentration, were analyzed in low levels (− 1) and high levels (+ 1) with both methods according to the Table S1. The four factors selected were tested with eight experiments designed in accordance with Heyden et al. (2001). The slopes obtained in standard curves of **1** were compared with the slopes obtained in standard curves of spiked compound **1** in each matrix. Three standard curves were obtained, in three consecutive days, by plotting the peak area versus the standard concentrations of **1** (0.1, 0.5, 1, 2.5, 5, and 7.5 μg ml^{-1}) in acetonitrile 50% (v/v) and in the matrix solutions. Five replicates were analyzed for each concentration level. The matrix effect was calculated based on the ratio of the peak area in the presence of matrix to the peak area in the absence of matrix, following the equation $ME\% = 100 \times [1 - (S_m/S_s)]$, where S_m indicates the slopes of the standard curves of **1** and S_s indicates the slopes of the standard curves of **1** in the matrix (European Medicines Agency 2012). For the system suitability, the following chromatographic parameters were calculated: time retention, theoretical plates (N), resolution (Rs), and tailing factor (T).

Recovery of Coumarins

Before the extraction procedure of porcine skin layers, the matrices were spiked with stock solution of **1** at concentrations 0.1, 0.5, 2.5, and 5 μg ml^{-1}. After that, 2 ml of MeOH was added to each matrix test tube, and the samples were maintained in an ultrasound bath for 45 min, filtered, and analyzed by HPLC and UFLC. The stability of matrices spiked with **1** was determined after 48 h of storage at room temperature. The analysis of these solutions was compared to freshly prepared solutions.

Method Application

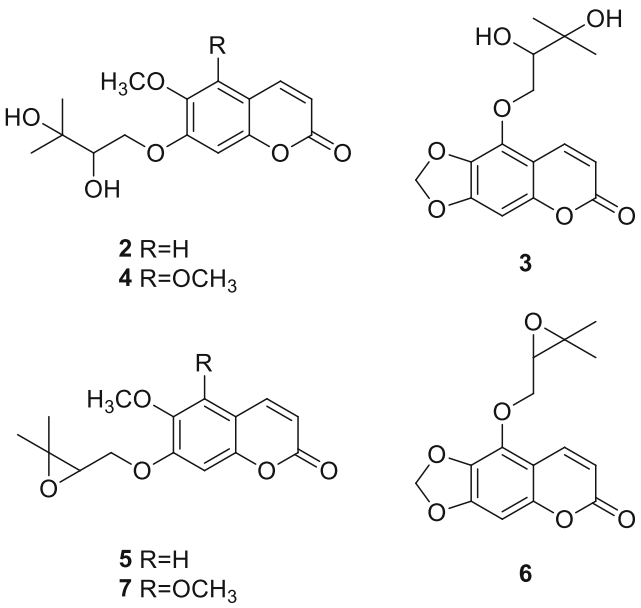
For permeation studies, nanoemulsions containing compound **1** (NE_{5MMDC}) or aqueous extracts (NE_{AE}) were prepared with a final content of 0.50 mg ml^{-1} (w/w). The positive control was a dispersion of **1** in propylene glycol. To determine the content of **1**, an adequate aliquot was diluted in acetonitrile 50% (v/v), filtered, and analyzed. The mean droplet size and polydispersity index of the nanoemulsions were evaluated through photon correlation spectroscopy at 25 °C in a Zetasizer NanoZS90 (Malvern Instruments, ENG) equipment. In the same equipment, the zeta potential was determined through electrophoretic mobility. The permeation/retention of **1** from NE_{5MMDC} and NE_{AE} nanoemulsions was evaluated using Franz-type diffusion cells. Porcine skin circular sections were dipped in phosphate buffer pH 7.4 solution for 15 min. After hydration, the skin was placed on the top of the cell. The acceptor medium was phosphate buffer:EtOH 60:40 (v/v). The cells were maintained in thermostatic bath at 32 ± 1.0 °C and stirred at 28×g force. Around 500 μl of formulations (NE_{5MMDC} and NE_{AE}) was placed on the donor compartment maintaining the sink conditions. The concentration of **1** was determined in the skin and in the acceptor phase after 8 h. The amount of coumarin retained in the skin was quantified through HPLC and UFLC after extraction of **1** with 2 ml of MeOH and submitted to sonication for 45 min. Results were expressed in micrograms of **1** per skin area.

Results and Discussion

UFLC Method Optimization

Figure 1 shows a typical HPLC/UV chromatogram for the replicated coumarins (**1**–**7**) in the analyzed aqueous extract. The coumarin content was estimated based on the linearity curve for the isolated chemical marker, compound **1** (Medeiros-Neves et al. 2015). The method was adapted by changing the acetic acid to formic acid due to its compatibility in various LC techniques (HPLC, UFLC, UPLC) coupled with an UV detector and a mass spectrometer. The conditions shown by the HPLC chromatogram (Fig. 1) provided an initial condition (method 1) for UFLC, shown in Fig. 2. However, the transfer method from an HPLC conventional column (~ 5 μm) to a UFLC column presenting a particle diameter of 2–3 μm must consider some other analytical modifications, such as adjustment of the temperature of the column oven. High oven temperatures provide a reduction in viscosity of the mobile phase, allowing the system to maintain a reasonable pressure, resulting in a faster analysis.

The initial approach, called method, 1 consists of flow rate of 0.45 ml min^{-1} using a gradient of 0–17% B (0–0.01 min), 17–20% B (0.01–2.80 min), 20% B (2.80–4.20 min), 20–25% B (4.20–5.60 min), 25–27% B (5.60–6.30 min), 27–30% B (6.30–7.00 min), 30–35% B (7.00–8.40 min), 35% B (8.40–9.80 min), and 35–17% B (9.80–11.20 min) with an oven temperature of 55 °C, an injection volume of 5 μl, and an analysis time of 12.6 min. These instrumental conditions did not allow the detection of all peaks (**1**–**7**) in the aqueous extract matrix. Method 2 used 0.55 ml min^{-1} flow rate with a minor change at the beginning of the gradient, which was increased to 0–17% B (0–0.05 min), maintaining the same conditions of oven temperature, injection volume, and analysis time of method 1. In method 2, we observed the presence of all coumarins in the aqueous extract matrix, which corresponded to following coumarins: 5-methoxy-6,7-methylenedioxycoumarin (**1**, chemical marker), 6-methoxy-7-(3′-methyl-2′,3′-dihydroxybutyloxy) coumarin (**2**), 5-(2′,3′-dihydroxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin (**3**), 5,6-dimethoxy-7-(3-methyl-2′,3′-dihydroxybutyloxy)coumarin (**4**), 6-methoxy-7-(2′,3′-epoxy-3-methylbutyloxy)coumarin (**5**), 5-(2′,3′-epoxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin (**6**), and 5,6-dimethoxy-7-(2′,3′-epoxy-3-methylbutyloxy)coumarin (**7**). However, this second method was not totally in accordance with the recommendations for the analyzed chromatographic parameters: number of theoretical plates (> 2000), tailing factor (< 1.5), and especially the factor resolution between peaks (> 1.5), while peak **3′** to the peak **1** showed a Rs < 1.5 and the peak **5′** and peak **6**, having a Rs < 1. Method 3 showed the best optimization results since all coumarins (**1**–**7**) were dereplicated from the aqueous extract in a short-time analysis (7 min) with an excellent factor resolution (Rs > 1.5): gradient elution was 0–17% B (0–0.09 min), 17–25% B (0.09–1.45 min), 25% B (1.45–2.20 min), 25–32% B (2.20–2.90 min), 32–37% B (2.90–4.77 min), 37–39% B (4.77–4.90 min), 39–40% B (4.90–5.60 min), 40% B (5.60–7.20 min), 40–22% B (7.20–7.80 min), 22–17% B (7.80–8.00 min). The flow rate was 0.55 ml min^{-1} up to 8 min, the wavelength was adjusted to 327 nm, the injection volume was 5 μl, and the analysis was carried out at 55 °C.



Method Validation

Table S2 compiles all parameters of system suitability for both HPLC and UFLC methods for the determination of compounds **1**–**7** in aqueous extract of the analyzed plant material. There was no matrix interference, showing that the peaks of coumarins were free from any substance that may be co-eluted, demonstrating that the proposed method is specific for the simultaneous analysis of coumarins **1**–**7** in all analyzed matrices in a short-time analysis (7 min). The standard linearity curve exhibited an excellent linearity and satisfactory determination coefficient over the given range of 0.1–7.5 μg/ml of **1**. The LOQ was close to 0.09 μg/ml for all matrices. The linearity data of the standard and the matrix effect for each matrix studied by the methods for HPLC and UFLC is presented in Table 1.

In relation to the repeatability and intermediate precision, relative standard deviation values (RSD%) were lower than 2% for all experiments on the same day or three different days (Table 1). The accuracy results for **1** were within the 99.31 to 102.04% range for the HPLC, and the 102 to 106% range for UFLC. Despite the complexity of the different matrices, the

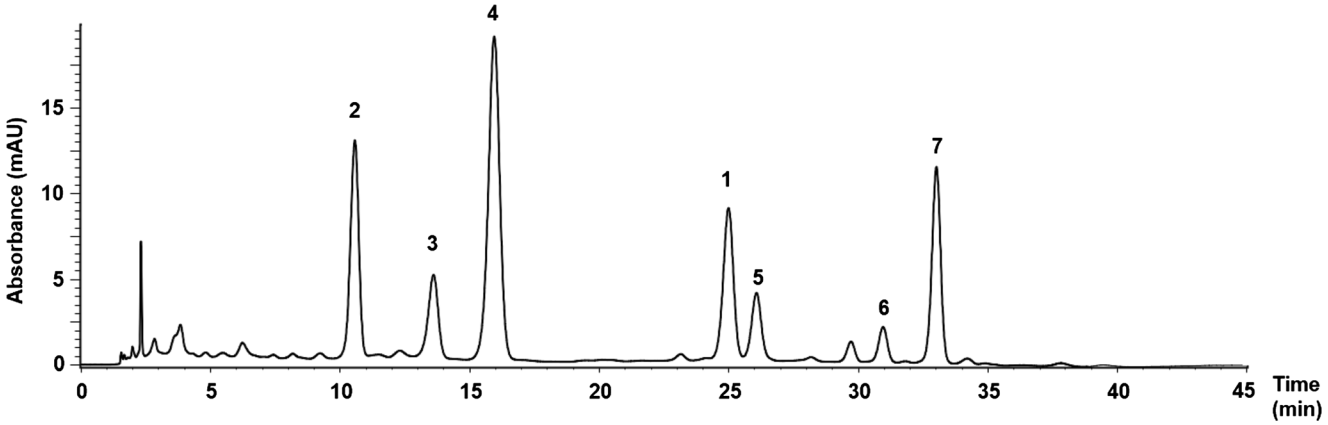


Fig. 1 Chromatographic profile of coumarins from the aqueous extract of *Pterocaulon balansae* obtained by the HPLC method. Chromatographic conditions: 0–17% B (0–0.01 min), 1 ml min^{-1} for 45 min at 30 °C with the injection volume of 20 μl

HPLC and UFLC methods can be considered precise and accurate according to official guidelines (ICH 2005). A multivariate approach using design of experiments is often recommended in robustness testing since several different factors can be analyzed concurrently with a reduced number of experiments. As shown in Fig. 3, in both methods, no significant factors were revealed for all analyses as the calculated *t*-values were lower than the critical *t*-values ($\alpha = 0.05$). Thus, there were no significant changes in the results in terms of the content percentage for **1** with the changes made in the

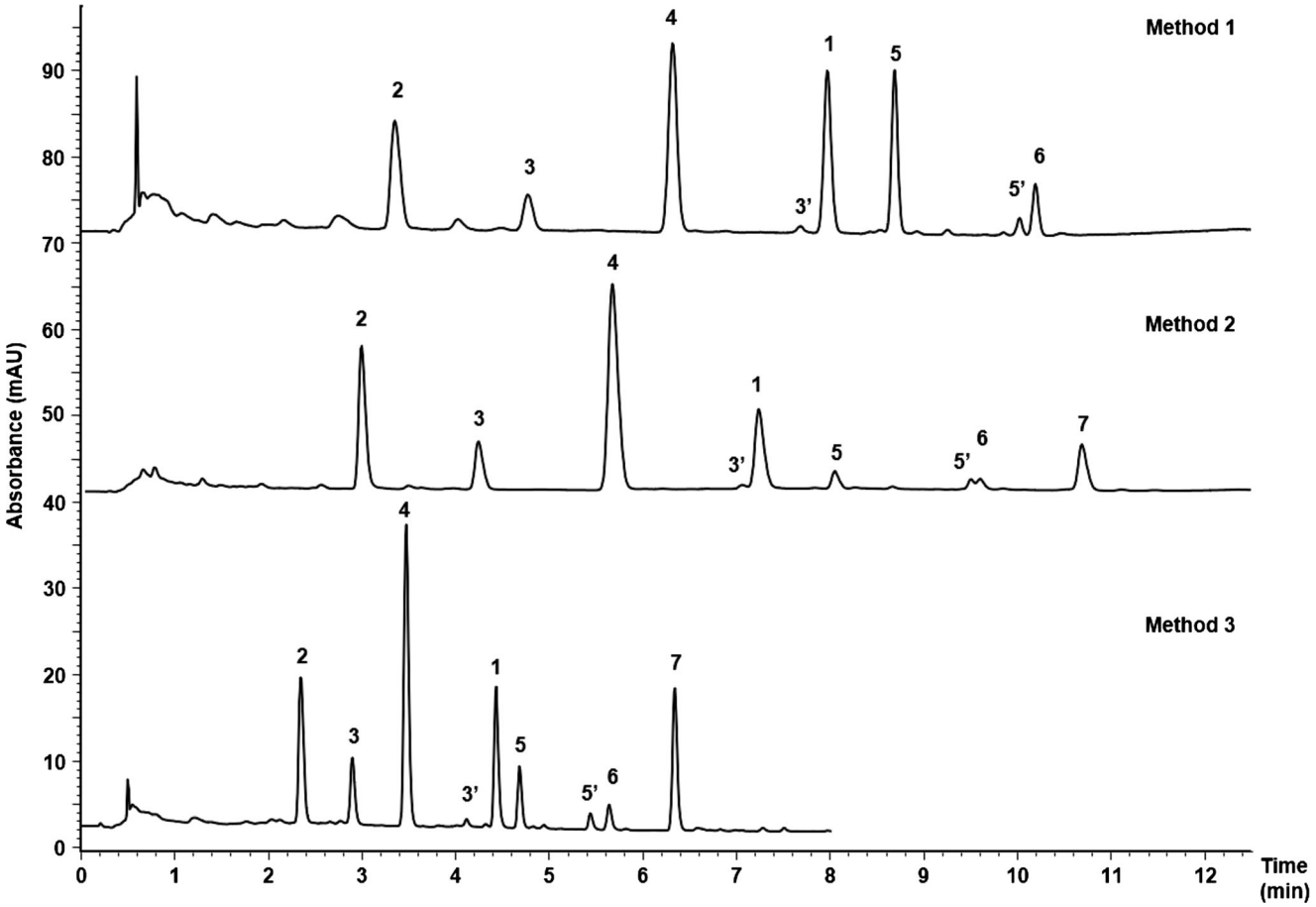


Fig. 2 Chromatographic profile of coumarins from the aqueous extract of *Pterocaulon balansae* by UFLC in the development of the method, where method 1: 0.45 ml min^{-1}, initial elution gradient of 0–17% B, oven temperature 55 °C, injection volume of 5 μl, and analysis time of 12.6 min; method 2: 0.55 ml min^{-1}, was increased to 0–17% B (0–0.05 min); and method 3: 0.55 ml min^{-1} up to 8 min, injection volume 5 μl, and the analysis was carried out 55 °C. The peaks **3′** and **5′** correspond to coumarins, with maximum UV absorption at 244/336 and 245/333, respectively. However, these compounds are in low amount in the aqueous extract for a complete structural elucidation

Table 1 Linearity data of the standard and the matrix effect for each matrix studied by the methods for HPLC and UFLC

		Standard	AE	NE_B	P. E/D	P. SC	RF
HPLC	Equation	$y = 52{,}077x + 478.22$	$y = 46{,}753x + 279.21$	$y = 47{,}487x + 312.44$	$y = 54{,}073x + 521.73$	$y = 49{,}406 + 489.28$	$y = 52{,}533 + 489.28$
	R^2	0.999	0.998	0.997	0.999	0.997	0.997
	LOD	0.030	0.021	0.034	0.052	0.022	0.022
	LOQ	0.092	0.095	0.088	0.091	0.093	0.093
	ME (%)	-	− 11.39	− 9.67	3.69	− 5.41	0.87
UFLC	Equation	$y = 13{,}142x + 120.20$	$y = 11{,}137x + 131.40$	$y = 11{,}973x + 420.40$	$y = 13{,}611x + 120.45$	$y = 12{,}249x + 131.40$	$y = 13{,}245x + 340.40$
	R^2	0.999	0.998	0.998	0.998	0.996	0.995
	LOD	0.030	0.022	0.025	0.034	0.031	0.023
	LOQ	0.091	0.094	0.092	0.093	0.090	0.091
	ME (%)	-	− 11.96	− 9.97	3.44	− 5.57	0.78

AE, aqueous extract of *Pterocaulon balansae*; *NE_B*, blank nanoemulsion; *P. E/D*, porcine epidermis/dermis; *P. SC*, porcine stratum corneum after tape stripping process; *RF*, receptor fluid; R^2 , determination coefficient; *LOD*, limit of detection; *LOQ*, limit of quantification; *ME*, matrix effect

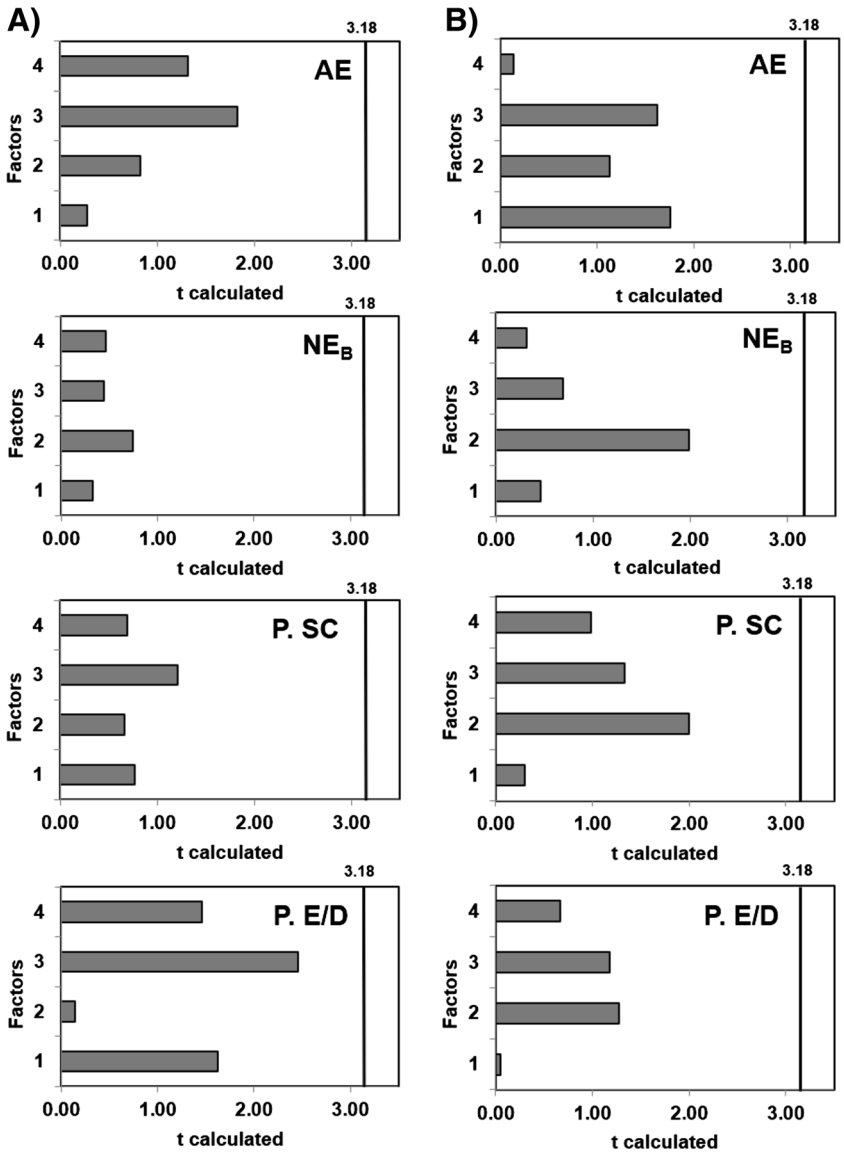


Fig. 3 Bar charts representing the *t*-calculated for quantitative determination (assay) of the investigated factors (1, 2, 3, and 4) in Plackett-Burman experimental design and their *t*-critical, represented by the vertical line. Column **A** represents the HPLC method and **B** the UFLC method, where 1, column oven temperature; 2, initial flow rate; 3, AF concentration; 4, initial organic composition; AE, aqueous extract of *Pterocaulon balansae*; NE_B, blank nanoemulsion; P. E/D, porcine epidermis/dermis; P. SC, porcine stratum corneum after tape stripping process

experimental conditions, thereby demonstrating the robustness for the proposed methods. The matrix effects for compound **1** are also presented in Table 1. The data indicate that samples exhibited low matrix effects in HPLC (ME% > − 11.39) and UFLC (ME% > − 11.96). The evaluation of the stability showed that the concentration of **1** in different matrices that remained constant after storage at 25 °C for 48 h varied from 99.45 to 101.99%. The recovery data for the quantification of **1** after extraction of previously spiked matrices are shown in Table S3 and were within FDA recommendations for bioanalytical method validation. The recovery yields are satisfactory and demonstrated that 45 min was sufficient for the complete extraction of **1** from matrices, and no matrix components interfered during the procedure with an adequate precision in all assessed matrices (RSD < 8.83%).

The suitability of the system was verified by standard substance routine analysis carried out on the experimental conditions with compound **1**. The obtained values for the parameters and variability (RSD%) for compound **1** in each method are HPLC: retention time 24.97 (0.04); theoretical plates 18,592 (0.94); tailing factor 0.96 (0.10); resolution between peaks **3′** and **1** Rs = 2.75 (0.61); resolution between **1** and peak **5** Rs = 1.51 (0.44) and UFLC: retention time 4.43 (0.08); theoretical plates 32,751 (1.59); tailing factor 1.38 (0.79); resolution between peaks **3′** and **1** Rs = 3.45 (1.33); resolution between **1** and peak **5** Rs = 2.62 (0.42). All values for the system suitability parameters for dereplicated compounds **1**–**7** are summarized in Table S2, which indicate that the UFLC methodology is suitable for a rapid analysis of coumarins.

Table 2 Determination of compound **1** in real samples

Application of methods		Mean of six replicates (RSD)	
		HPLC	UFLC
Samples			
NE_{5MMDC} (mg ml^{-1})		0.45 (1.51)	0.46 (1.67)
NE_{AE} (mg ml^{-1})		0.46 (1.45)	0.47 (1.34)
Skin retention			
Total skin (μg cm^{2-1})	NE_{5MMDC}	2.84 (17.46)[a]	2.82 (16.49)[a]
	NE_{AE}	3.02 (15.86)[a]	3.00 (18.24)[a]
RF (μg cm^{2-1})	NE_{5MMDC}	0.46 (12.94)[b,c]	0.43 (11.76)[b,c]
	NE_{AE}	0.27 (9.82)[b,c]	0.28 (14.82)[b,c]

[a] NE_{AE} and NE_{5MMDC} were statistically different ($p < 0.05$) from the control in skin retention

[b] NE_{AE} and NE_{5MMDC} were statistically different ($p < 0.05$) in permeation

[c] NE_{AE} and NE_{5MMDC} were statistically different ($p < 0.05$) from the control in permeation

RF, receptor fluid; *NE_{5MMDC}*, 5-methoxy-6,7-methylenedioxycoumarin-loaded nanoemulsion; *NE_{AE}*, aqueous extract of *Pterocaulon balansae*–loaded nanoemulsion; *RSD*, relative standard deviation

Application of the Method

Next, the nanoemulsions (NE_{AE} and NE_{5MMDC}) were analyzed and yielded monodisperse emulsions (polydispersity index < 0.15) with mean droplet size of 235 nm and negative ζ-potential of − 23 mV. Such physicochemical properties follow in line with our previous results for formulations containing extracts and/or fraction compounds obtained from medicinal plants (Bidone et al. 2015; Fasolo et al. 2009). The total amount of **1** in NE_{AE} was close to 0.5 mg ml^{-1}, showing no loss of coumarins during preparation (Table 2). Similar results were noticed for a control formulation (NE_{5MMDC}) in terms of physicochemical properties and content of compound **1**. The skin permeation profile for compound **1** was evaluated from AE-loaded (NE_{AE}) and compound **1**–loaded (NE_{5MMDC}) nanoemulsions using Franz-type diffusion cells. After 8 h, the retention of **1** from NE_{AE} and NE_{5MMDC} was significantly different ($p < 0.05$) from the control with propylene glycol, however, without statistical differences among formulations. The amount of **1** retained on skin was approximately 3 μg/cm^2 for both formulations in both methods (Table 2). The permeation of **1** from formulations was also significantly different ($p < 0.05$) from the control. However, the amount of **1** which permeated through the skin from NE_{AE} was significantly lower ($p < 0.05$) (0.27 ± 0.03 μg/cm^2) in comparison with the formulation containing the isolated coumarin (0.46 ± 0.06 μg/cm^2). These preliminary results suggest that the amount of **1** retained in the skin was not influenced by the presence of the other components in the extract; however, the amount of **1** permeated was lowered in the presence of these compounds. Table 2 presents the results for the quantification of compound **1** by both analyzed HPLC and UFLC methods in nanoemulsions NE_{AE} and NE_{5MMDC} in amount of approximately 0.46 mg ml^{-1} in both formulations. The values for compound **1** were not significantly different ($p > 0.05$) between both methods.

Conclusions

This study showed the development and validation of LC methods for the determination of coumarins from aqueous extracts of *P. balansae* in analytical and bioanalytical samples. The determination of coumarins was based on analytical validation carried out for 5-methoxy-6,7-methylenedioxycoumarin (**1**), a chemical marker for medicinal plant members of the genus *Pterocaulon* from the composite or sunflower family. Both HPLC and UFLC proved to be linear, precise, accurate, and robust to estimate coumarins in different matrices. However, the UFLC method was faster (almost fourfold) and consumes less eluent being more eco-friendly in comparison with traditional HPLC.

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Authors' Contributions BMN contributed to plant collection and identification, confection of herbarium, running the laboratory work, analysis of the data, and drafting the paper. MCN and NTB contributed to the laboratory work and analysis of the data. RSS and MS contributed to critical reading of the manuscript. HT and GVP designed the study, supervised the laboratory work, and contributed to critical reading of the manuscript. All authors have read the final manuscript and approved the submission.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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