Talanta 101 (2012) 530–536

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Development and validation of a stability indicative HPLC–PDA method for kaurenoic acid in spray dried extracts of *Sphagneticola trilobata* (L.) Pruski, Asteraceae

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ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form
11 September 2012
Accepted 14 September 2012
Available online 25 September 2012

Keywords:
Sphagneticola trilobata
Kaurenoic acid
Analytical validation
Liquid chromatography
Spray dried extract
Stability indicative method

ABSTRACT

A gradient stability indicative HPLC–UV method was developed and validated for assay of the marker kaurenoic acid (KA) in spray dried extract of *Sphagneticola trilobata* (L.) Pruski. The marker, and another unidentified polar component, were separated on a Luna Phenomenex C_{18} column (250 × 4.6 mm, 5 μm) with mobile phase composed of acetonitrile:acidified water pH 3.0 with phosphoric acid, in a gradient run of 40 min; at a flow rate of 1.0 mL min^{-1}, 35 °C, using wavelengths of 210 and 338 nm. The method was linear over a KA concentration range of 4.5–30.0 μg mL^{-1}, without interference of the herbal matrix on the linearity of the method. The RSD% values for the intra- and inter-day precision studies were < 2.0 and < 8.0% for inter-laboratorial study. The method showed excellent KA recovery (99.0%). The LOQ value was found to be 1.13 μg mL^{-1} and the method proved to be robust for small, deliberate changes in temperature and pH of the mobile phase with RSD% < 2.5% for the KA assay. A forced degradation study of *S. trilobata* dried extract was conducted under conditions of visible light (1.200.000 l × h^{-1}) and UV (200 Whm^{-2}) irradiation, acid (0.5 mol L^{-1} HCl, 30 min), basic (1 mol L^{-1} NaOH, 2 h) and oxidative (30% H_2O_2, 4 h) hydrolysis, in order to develop a gradient stability-indicating LC–UV method for KA quantification, the selected marker, and also to detect the major polar components of the extract, under investigation. The KA contents remaining after these stress conditions were 72.3, 70.0, 97.6, 65.8 and 87.0%, respectively. The alkaline conditions resulted in higher degradation for the unknown polar components of the extract, without interference of supplementary peaks at the retention time of the KA. This method can be used for the KA assay and qualitative analysis of polar components in stability study of spray dried extracts of *S. trilobata*, for subsequent use in the quality control of dosage forms.

1. Introduction

Sphagneticola trilobata (L.) Pruski is a member of the Asteraceae family, and its synonyms include *Acmella brasiliensis* and *Wedelia paludosa*. It is a creeping plant, native to the tropics of Central America, and has naturalized in many humid tropical areas of the world, including Brazil, where it is known as *pseudo-arnica, margaridão* or *picão da praia* [1]. According to information from the Missouri Botanical Garden, there is a higher frequency of records of the plant in Central America [2]. In folk medicine, *S. trilobata* is used to treat backache, muscle cramps, rheumatism, stubborn wounds, sores and swellings, and painful arthritic joints [3].

Our research group has demonstrated the presence of the diterpene kaurenoic acid (ent-16-kauren-19-oic acid), the flavonoid luteolin (5,7,3′,4′-tetrahydroxi flavone) [4], the chalcone coreopsin [5], a new eudesmanolide lactone denominated paludolactone [6] and stigmasterol [7].

Kaurenoic acid (KA), exhibits interesting biological properties, including analgesic [4,7], trypanosomicidal [8], antifungal [9], smooth muscle relaxant [10], anti-inflammatory effects [11–14] and hypoglycemic properties [15]. The latter authors showed the higher concentration of KA in the roots and stems during the autumn.

Although several studies on the chemical composition and pharmacological activity of the Genus *Wedelia* can be found in literature [4–8,16–22], there are few analytical methods for qualitative and quantitative analysis of its plant extracts and derivatives. Batista et al. [23] used an LC–UV isocratic method for

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http://dx.doi.org/10.1016/j.talanta.2012.09.024

analysis of extract and fractions of *S. trilobata*, in order to quantify the kaurenoic and grandiflorenic acid, but the method did not separate the polar components of the extract, and showed poor resolution between KA and the neighboring peaks in the chromatogram. Bresciani et al. [15,24] used GC-FID for KA quantification in the different parts of *S. trilobata*, which is an impractical method for quality control in routine use. Therefore, pharmacological studies with the extracts derived from the aerial parts of *S. trilobata* indicate its potential use in the treatment of pain and inflammation, requiring the development and validation of methods for quality control of the extracts and their standardization. To the best of our knowledge, the stability of KA and the overall spray dried extract of *S. trilobata* have not yet been reported in the literature. The International Conference on Harmonization (ICH) drug stability test guideline Q1A requires that analysis of stability samples be done through the use of validated stability-indicating analytical methods [25], but this concept is often used in synthetic drugs and is not exploited in herbal drugs. The results of stress tests often help guide the pharmaceutical dosage form (i.e. gastro resistant coating of tablets), and provide information about the instability of drugs, enabling the development of stability-indicating analytical methods that may be used in the subsequent stability studies on the active ingredient.

The present work, therefore, sought to develop and validate a stability indicative LC–UV method for the determination of KA and qualitative analysis of polar components (fingerprinting) in aerial parts of spray dried extracts of *S. trilobata*, for subsequent use in the quality control of dosage forms, aimed at the future development of a new anti-inflammatory topical herbal medicine.

2. Experimental

2.1. Reagents and standards

All the solvents were HPLC grade (Tedia, Fairfield, Ohio, USA) and were degassed by helium gas. The water was purified using a Milli-Q system (Millipore, Massachusetts, USA). All the solutions were filtered through a 0.45 μm membrane (Millipore, Massachusetts, USA). Kaurenoic acid was isolated from the roots of *S. trilobata.* Briefly, the dried and milled roots were extracted with acetone (1:5 w/v) by static maceration for 7 days at room temperature and then filtered through filter paper. The solution was evaporated (at 45 °C) and submitted to liquid–liquid extraction using hexane three times. After evaporation of the organic solvent, the material was submitted to fractionation by open column chromatography using silica gel 60, as stationery phase and hexane:ethyl acetate mixtures with increasing polarity 99:1–85:15 (v/v) followed by ethanol as mobile phase. The KA was then purified by recrystallization in hexane and characterized in comparison with an authentic sample by Infra-Red Spectroscopy (FTIR), TLC, HPLC, melting point and Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR) [26] showing purity of 95.0% by HPLC.

2.2. Plant materials

Aerial parts of native specimens obtained from vegetative propagation of authentic *S. trilobata* were collected in Itajaí (Santa Catarina, Brazil) in February 2007 and identified by Prof. Rene Ferreira. The plant was collected after six months of cultivation (time set in the harvesting study—unpublished data), dried at 35 °C in a circulating air oven, and stored in polyethylene bags covered with paper. A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC, Brazil) under number V.C. Filho, 002.

2.3. Preparation of hydroalcoholic extract

Three batches of dried extracts were prepared at Centroflora (Botucatu-SP, Brazil) on a pilot scale (1 kg). The aerial parts were milled (sieving 2 mm) and submitted to dynamic maceration with ethanol–water 60:40 (v/v) at a plant:solvent ratio of 10:90 (w/v), stirred for 8 h at room temperature, and then filtered through a Nutschell filter with filter paper. The solution was concentrated in a Bernhauer concentrator at 70 °C under vacuum (20–25 bar, at 4.00 kgf cm^{-2}), to obtain total solids of approximately 25% (concentrated extract). The concentrate was mixed with about 15% (w/w total solids of concentrate) of colloidal silicon dioxide and dried in an industrial spray dryer (GEA-Niro, Søborg, Denmark) with an inlet temperature of 150–170 °C and an outlet temperature of 70–80 °C, to obtain the dried extract.

2.4. HPLC analysis

A Shimadzu Proeminence LC-20AT LC system (Shimadzu, Tokyo, Japan), consisting of a binary pump and a Shimadzu SPD-M20A photodiode array detector, SIL-20AHT auto-sampler, DGU-20A5 in-line degasser and software Class VP (version 6.14) were used. The injections (20 μL) were carried out on a Phenomenex (Torrance, California, USA) Luna C18 5 μm (250 × 4.6 mm) conditioned in a Shimadzu CTO-10AS VP column oven equilibrated at 35 °C, with detection at 210 and 338 nm. For the method development, different solvent systems were assayed in isocratic and gradient conditions using methanol, acetonitrile and acidified water (pH 3.0 with phosphoric acid), at 1.0 mL min^{-1}. The best gradient was chosen: gradient acetonitrile (A):acidified water pH 3.0 with (B): phosphoric acid of 15:85 (A:B) (0 min); 20:80 (5 min); 22:78 (8 min); 25:75 (12 min); 30:70 (20 min); 60:40 (22 min); 90:10 (25 min) maintaining this composition until 40 min then returning to the initial conditions for a further 10 min.

At least five individual injections of KA Standard Solution were performed before all the measurements, to assess the suitability parameters, including resolution (R) between KA and neighboring peak of $R > 1.0$, tailing factor (T) of KA < 1.5 and repeatability of the KA peak area (RSD% < 2.0).

2.4.1. Sample preparation

Dried extracts were diluted at 2 mg mL^{-1} in methanol:water (80:20 v/v), submitted to sonication for 30 min. The solution was filtered through a 0.45 μm modified PTFE membrane (Millex®), prior to injection into HPLC.

2.4.2. Standard solution

Kaurenoic acid (7.5 mg with 95% purity) was dissolved in 50 mL of methanol, sonicated for 30 min (150 μg mL^{-1}), and used for the analytical validation. This solution was diluted 1:10 with methanol:water (80:20 v/v) to produce a working solution (15 μg mL^{-1}) for assay of the samples. Both solutions were freshly prepared and filtered through a 0.45 μm modified PTFE membrane (Millex®), prior to injection into the chromatograph.

2.5. HPLC–UV method validation

The method was validated according to the ICH guidelines (2005) and the Brazilian legislation [27].

2.5.1. Selectivity and degradation tests

The selectivity of the HPLC method was evaluated by comparing the chromatogram of a blank (methanol:water 80:20 v/v), the mobile phase, and the sample solution, to detect any co-elution

interferents. To verify whether the method was stability-indicating of the selected marker (KA), and also for the other major components of the dried extract, forced degradation was performed until 5–15% of marker degradation [29], or less in the case of over degradation of other unidentified major components. The dried extract (100 mg) was submitted to stress tests under acidic conditions (10 mL of 0.5 mol L^{-1} hydrochloric acid—HCl, 30 min), alkaline conditions (10 mL of 1.0 mol L^{-1} sodium hydroxide—NaOH, 2 h) or oxidative conditions (10 mL of 30% H_2O_2, 4 h), all at 25 °C, under magnetic stirring. In all cases, after the addition of the solvent degradation, a further 10 mL of methanol:water (80:20 v/v) was added, followed by 30 min of sonication. The flask was then filled to volume (volumetric flask of 25 mL) with the latter solvent. After the degradation time had elapsed, 5 mL was transferred to another 10 mL volumetric, neutralized, and the volume completed with methanol:water (80:20 v/v). In addition, the dried extract was also submitted to visible light (1.200.000 $l \times h^{-1}$) and UV (200 Wh m^{-2}) in a photostability chamber (Mecalor, EC/0,2/AR-F, São Paulo, Brazil) and the sample solution was prepared as described in Section 2.4.1. After filtration through a 0.45 μm modified PTFE membrane, the degraded samples were injected into the chromatograph, comparing with a fresh, non-degraded sample solution (see Section 2.4.1).

2.5.2. Linearity, LOD and LOQ

Linearity was evaluated for analytical curves using two different procedures. In the first procedure, KA Standard Solutions diluted in the range of 4.5–30 μg mL^{-1} were used. Three sets of eight different calibration solutions were injected into the chromatographic system in a single injection, after filtration through a 0.45 μm modified PTFE membrane.

The influence of the matrix in the linearity of KA was evaluated by spiking a sample solution (at 5 mg mL^{-1}, prepared as described in Section 2.4.1) with increasing volumes of a concentrated KA standard solution (at 150 μg mL^{-1} prepared as described in Section 2.4.2), corresponding to 30%, 50%, 70%, 90%, 100%, 120%, 170% and 200% of KA theoretical value (15 μg mL^{-1}). These solutions were prepared in triplicate, and injected into the chromatographic system after filtration through a 0.45 μm modified PTFE membrane. A 24-point analytical curve was plotted and statistically evaluated for each solution.

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were mathematically determined through the calibration curve. The aforementioned factors (3.0 and 10, for LOD and LOQ, respectively) were multiplied by the standard deviation of the linear coefficient and divided by the slope, according to the guidelines[27,28].

2.5.3. Accuracy

The accuracy of the method was measured through the analyte recovery test [27,28] in triplicate. Standard concentrations of about 10.5, 15 and 22.5 μg mL^{-1} for KA were added to the sample solution. The sample (50 mg) was sonicated for 30 min with 40 mL of methanol:water (80:20 v/v) in a 50 mL volumetric flask. Before completing to volume, 1, 2.5 and 5.0 mL of KA standard solution were added (at 150 μg mL^{-1}, prepared as described in Section 2.4.2). After spiking, the volume was completed with the same solvent. The recovery of KA was calculated after discounting the analyte area present in the sample solution without the addition of KA. The solutions were prepared in triplicate and injected after filtration through a 0.45 μm modified PTFE membrane.

2.5.4. Precision

Repeatability (intra-day), intermediate precision (inter-day) and repeatability (inter-laboratory) were determined through analysis (triplicate) of the sample (spray dried extract) at three levels (1, 2 and 3 mg mL^{-1}), prepared as described in Section 2.4.1. The RSD% (relative standard deviation) of the KA assay was determined. The intermediate precision was determined over a period of two days and reproducibility was determined in two different laboratories, using the same homogeneous sample (spray dried extract).

2.5.5. Robustness

The robustness of the extraction method and chromatographic method, related to the *R* and assay of KA in the sample, was evaluated by changing: (i) the extraction time (sonication for 25, 30 and 35 min), (ii) the solvent composition (methanol:water, 75:25, 80:20 and 85:15; v/v), (iii) the mobile phase flow (0.9, 1.0, 1.1 mL min^{-1}), (iv) the mobile phase pH (2.7, 3.0 and 3.3), (v) the oven temperature of column (34, 35 and 36 °C), (vi) and (vii) the stability of analytical solutions (0, 24, 48 and 96 h). For each of the above conditions, the sample solution and the standard solution (prepared as described in Section 2.4.1 and Section 2.4.2) were injected (triplicate) after filtration through a 0.45 μm modified PTFE membrane. The data were evaluated using single-factor analysis of variance (ANOVA) ($p < 0.05$).

3. Results and discussion

A gradient HPLC–UV method was developed for qualitative analysis of the chromatographic profile of spray dried extract of *S. trilobata*, and validated for quantitative analysis of KA.

KA found in *S. trilobata* is important for its therapeutic effect, particularly its analgesic and anti-inflammatory effects. It is not available commercially, but is widely found in plants, and was isolated by our research group and characterized with purity of 95.0% using high performance liquid chromatography coupled with photodiode array detection (HPLC–PDA) (Fig. 1a). The supplementary peaks that appear early on are “ghost peaks”. These are also observed in the blank solution (Fig. 1(c), and may be due to: (a) the low wavelength chosen for detection of KA (210 nm); and (b) the wide organic solvent variation in the mobile phase (ACN:acidified water of 60:40 at 22 min to 90:10 at 25 min). This wide organic solvent range was necessary to detect both the polar components, which eluted until 22 min, and the hydrophobic components such as KA, which eluted from the column only at 38 min, at 90% of ACN (Fig. 1a, b). It is important to highlight the difficulty of developing a method for this kind of sample, in view of its UV absorption, the interference of solvents, and the range of polarity of the hydroethanolic spray dried extract of *S. trilobata*.

The method developed showed a relatively satisfactory resolution (*R*) of the marker (KA) from its neighboring peaks ($R = 1.3$) in the chromatogram of *S. trilobata* extract (Fig. 1b). At 338 nm, the KA is not observed, due to its spectral characteristics, and is visualized only the major polar components, which eluted until 22 min (Fig. 1b, d). Besides the KA quantification, the developed HPLC method highlighted the presence of four major peaks in the polar region of the chromatogram, which can be monitored in the study of stability of future plant derivatives. These unknown components in the polar region of the chromatogram presented a UV spectral typical of phenolic compounds, particularly flavonoids. Its isolation and structural elucidation are being investigated.

Fig. 1e–i. shows the degraded sample, after the acid (0.5 mol L^{-1} HCl, 30 min), alkaline (1.0 mol L^{-1} NaOH, 2 h), oxidative (30% H_2O_2, 4 h), visible light (1.200.000 $l \times h^{-1}$) and UV (200 Wh m^{-2}) stress tests, respectively. The KA residual contents (% in relation to the original assay, in the non-degraded sample), after these stress conditions, are 72.3%, 70.0%, 97.6%, 65.8% and 87.0%, respectively. In all the conditions used, the appearance of small, supplementary peaks was observed, as well

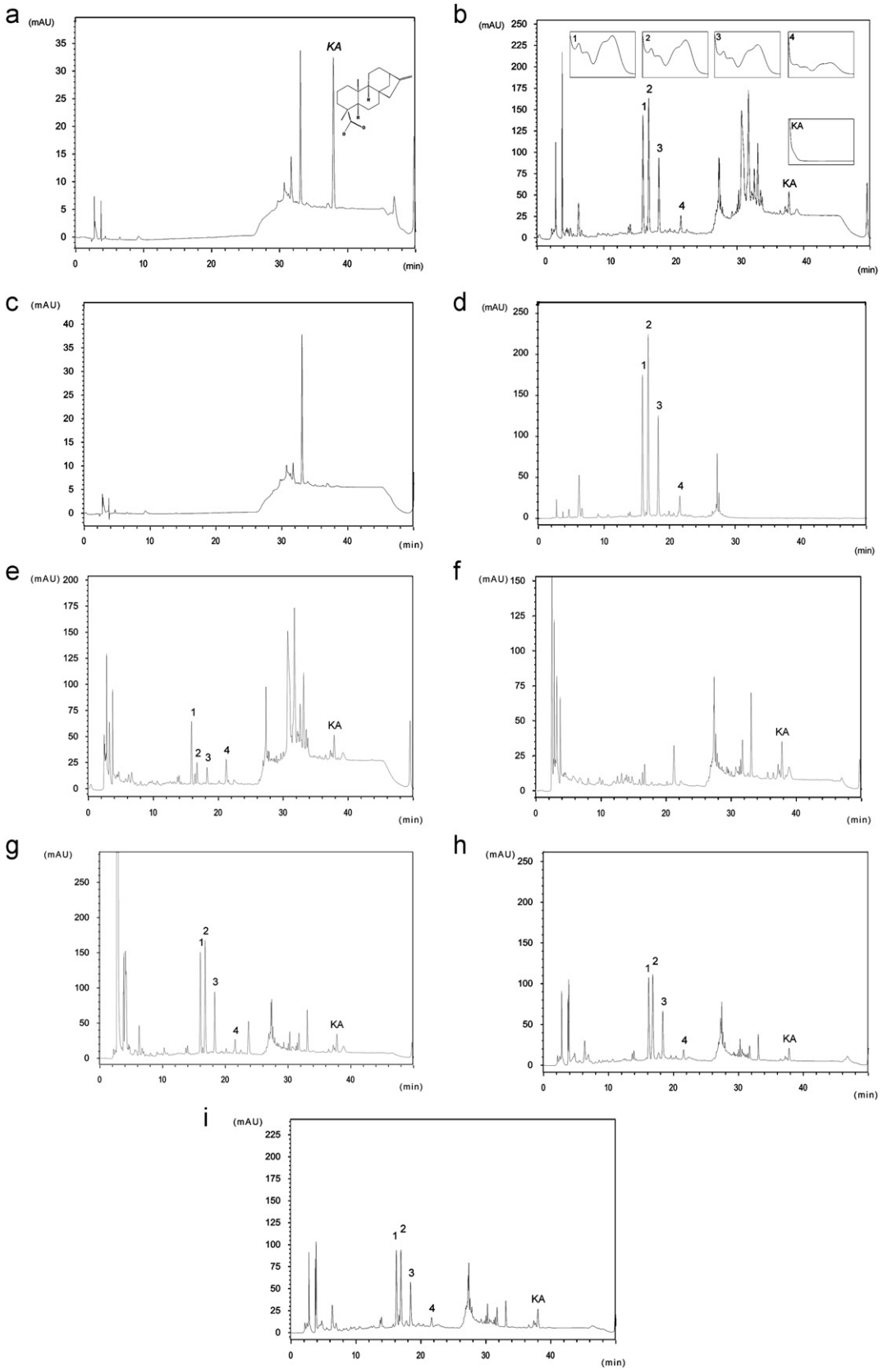

Fig. 1. Chromatographic profile at 210 nm of: (a) kaurenoic acid (KA) at 15 μg mL^{-1}; (b) sample solution of dried extract of *S. trilobata* at 2 mg mL^{-1} (UV spectra in the insert, with peak 5 corresponding to KA); (c) blank solution (methanol:water pH 3.0 80:20 v/v); (d) sample solution of dried extract of *S. trilobata* at 2 mg mL^{-1} at 338 nm; (e) dried extract of *S. trilobata* after stress with 0.5 mol L^{-1} HCl 30 min, at 210 nm; (f) dried extract of *S. trilobata* after stress with 1.0 mol L^{-1} NaOH 2 h, at 210 nm; (g) dried extract of *S. trilobata* after stress with 30% H_2O_2 4 h, at 210 nm; (h) dried extract of *S. trilobata* after stress with visible light 1.200.000 $l \times h^{-1}$, at 210 nm; and (i) dried extract of *S. trilobata* after stress with UV 200 Whm^{-2}, at 210 nm.

as a decrease in the marker peak, and also in the other unknown major components of the extract The alkaline conditions resulted in higher degradation for the polar unknown components of the extract and the appearance of additional peaks in other regions of the chromatogram (Fig. 1f). The UV absorption profile of these peaks was suggestive of phenolic compounds, which may explain the pronounced susceptibility of these compounds to alkali degradation. The degradation in acidic media provided no significant alteration in the chromatographic profile of the *S. trilobata* extract, only a decrease in the peaks of both the marker and the other compounds (1–4) in the polar region of the chromatogram (Fig. 1e). In the oxidative degradation test (Fig. 1g), the polar region of the chromatogram remained practically unchanged, with the appearance of an extra peak at 24 min, and the disappearance of peaks after the sudden increase of organic solvent in the mobile phase (28–34 min). A similar chromatographic profile was observed after visible and UV exposure of the sample (Fig. 1h and i), which may indicate a possible photolytic oxidation of these unknown compounds. In addition, compounds 1–4, as well as the marker, showed a decrease after visible and UV exposure. The possible degradation pathways of these compounds may be related to their oxidation, hydrolysis or isomerization, which is a subject that requires further studies. However, there was no interference in retention time of the AK, showing the selectivity of the developed method, which may be considered stability indicative for the marker in the spray dried *S. trilobata* extract, making it useful for future stability studies.

The KA calibration curve proved to be linear over the proposed range (4.5–30.0 μg mL^{-1}), as shown by linear regression coefficients (r^2) > 0.999 for both the isolated standard and the standard addition in the matrix (Fig. 2), demonstrating an acceptable data fit to the regression line. Statistical analysis of linear regression (*F* test) demonstrates the statistical significance of the linearity of the method. In addition, both curves (isolated KA and standard addition to the matrix) proved to be parallel, without interference of the matrix on the linearity of the method. Plotting the values in the residue graph, it was observed that a curvilinear effect fits into the quadratic model, and the observed *y* values are very close to the predicted y values [30].

The sensitivity of the method for KA was expressed as the slope of the analytical curve, and as the LOD and LOQ values of 1.05 and 3.5 μg mL^{-1}, respectively.

The accuracy of the standard in the spiked sample was evaluated at three levels: 10.5, 15 and 22.5 μg mL^{-1} for KA, with recoveries of about 99.0%, without statistical difference by the F test ($p > 0.05$) and RSD% < 3.0% in all determinations of the recovery test (Table 1). The method showed good accuracy at low, medium and high level concentrations for the marker, within the linearity of the method. This validation parameter shows good reliability for determining the level of the marker on the dry extract of *S. trilobata* extract.

Although there was a statistically significant difference (*t* test) at $p < 0.05$ and $p < 0.01$ for intermediate precision and reproducibility, respectively, the %RSD was less than 2.0% for both the intra (repeatability) and the inter-day (intermediate precision) study. The inter-laboratorial (reproducibility) study showed %RSD < 8.0%, considered acceptable [27], showing the high precision of the method, which can be reproduced in different laboratories with low variation in the results.

The robustness of the method was analyzed by small, deliberate variations in sample extraction time (25, 30 and 35 min), composition of solvent extraction (75:25, 80:20 and 85:15 of methanol:water v/v), oven temperature (34, 35 and 36 °C), mobile phase flow (0.9, 1.0 and 1.1 mL min^{-1}) and pH of aqueous phase (2.7, 3.0 and 3.3) as shown in Table 2. The robustness was estimated using the overall mean, standard deviation, %RSD and

Table 1
Accuracy and precision results of the HPLC method for kaurenoic acid (KA) assay in *S. trilobata* spray dryer extract.

Parameter	Level (%)		
Analytical parameter	50	100	150
Accuracy (% ± %RSD)	98.27 (2.8)	98.26 (1.2)	98.69 (2.5)
Repeatability (%RSD)	0.47	0.48	1.09
Intermediate precision (%RSD)	1.07	0.79[a]	1.83[a]
Reproducibility (%RSD)	6.63[b]	6.82[b]	7.29[b]

[a] $p < 0.05$.
[b] $p < 0.01$.

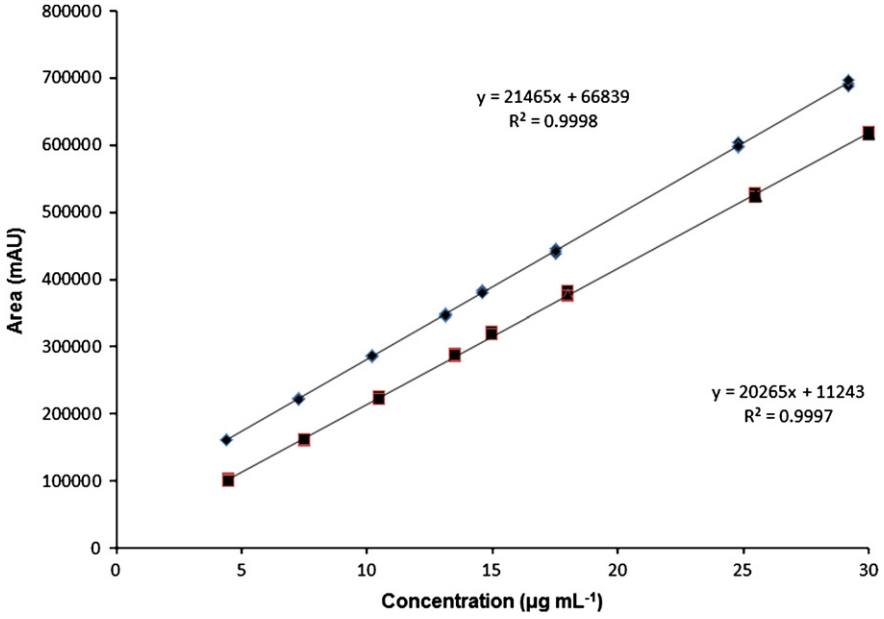

Fig. 2. Calibration curves of KA (squares) and of the standard addition in the vegetable matrix (triangles) at 210 nm

Table 2
Chromatographic parameters in the robustness tests of the HPLC method for kaurenoic acid (KA) assay in *S. trilobata* spray dryer extract.

Parameter	Average (%RSD_{intra})			
	Rt	Area	Resolution	Assay (mg/g)
Extraction time (min)				
25	37.9 (0.01)	271,149 (0.8)	1.3 (0.2)	6.4 (0.9)
30	37.9 (0.02)	270,837 (1.4)	1.4 (0.2)	6.4 (1.2)
35	37.9 (0.02)	269,236 (0.2)	1.4 (0.1)	6.4 (0.2)
%RSD_{inter}	0.02	0.9	0.1	0.8
t test	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Solvent of extraction (methanol:water)				
75:25	37.9 (0.01)	272,192 (0.2)	1.3 (0.2)	6.5 (0.05)
80:20	37.9 (0.02)	270,837 (1.4)	1.4 (0.2)	6.4(1.2)
85:15	37.9 (0.01)	270,892 (0.2)	1.3 (0.3)	6.4(0.2)
%RSD_{inter}	0.03	0.8	0.4	0.7
t test	$p < 0.01$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Flow (mL min^{-1})				
0.9	39.8 (0.04)	311,549 (0.1)	1.4 (0.1)	6.5 (0.1)
1.0	37.9 (0.03)	276,926 (0.3)	1.4 (0.4)	6.4 (0.3)
1.1	36.7 (0.01)	244,490 (0.3)	1.3 (0.4)	6.1 (0.3)
%RSD_{inter}	3.5	10.5	4.9	2.6
t test	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
Oven temperature (°C)				
34	37.9 (0.05)	277,388 (0.4)	1.4 (0.5)	6.4(0.4)
35	37.9 (0.03)	276,926 (0.3)	1.4 (0.4)	6.4 (0.3)
36	37.7 (0.03)	278,985 (0.2)	1.4 (0.3)	6.4 (0.2)
%RSD_{inter}	0.2	0.4	0.5	0.6
t test	$p < 0.01$	$p > 0.05$	$p > 0.05$	$p < 0.01$
pH of mobile phase				
2.7	37.9 (0.03)	270,699 (0.5)	1.4 (0.2)	6.4 (0.05)
3.0	37.9 (0.03)	274,070 (0.4)	1.4 (0.1)	6.5 (0.4)
3.3	38.0 (0.01)	274,130 (0.5)	1.4 (0.1)	6.4 (0.5)
%RSD_{inter}	0.05	0.7	0.8	0.8
t test	$p < 0.01$	$p < 0.05$	$p < 0.01$	$p < 0.05$

Rt: retention time; %RSD: relative standard deviation.

t test for area, retention time (Rt), *R* and assay of KA in the sample. Although statistically significant differences were observed in almost deliberate variations (except for extraction time), at $p < 0.05$ or $p < 0.01$, the method can be considered robust due to the low %RSD measured. In the sample extraction step, the method proved to be robust (%RSD < 1.0% for extraction time and solvent composition variations). The flow variation of mobile phase was the most impacting variable in the method, with %RSD close to 3.0% in the KA assay and statistical differences at $p < 0.01$ were observed for all the chromatographical parameters. The increase in flow decreases the Rt, Area, *R* and assay of KA. The method proved to be robust for temperature and pH changes, with %RSD < 2.5% for the assay of KA. The demonstration of robustness is essential in the transfer of the analytical process to other laboratories.

The selected conditions (35 °C, pH 3.0 and 1.0 mL min^{-1} flow) presented good results for KA analysis, as shown by the acceptable resolution ($R = 1.3$), peak asymmetry < 1.5, purity peak (0.99) using high performance liquid chromatography coupled with photodiode array detection (HPLC–PDA) and repeatability of area (RSD% < 2.0). While it is feasible to determine two peaks with $R < 1.5$, there will be some overlap of peak area [31]. Based on the valley between the KA peak the neighboring peak, a resolution of $R = 1.3$ was estimated for these two adjacent bands. Batista et al. [23] reported an isocratic method for analysis of KA and grandiflorenic acid from *S. trilobata* with $R = 0.98$. Thus, the present validated method is more selective and enabled a profile of other polar components of the extract under structural elucidation by our research group.

The validated analytical method was applied to three different batches of spray dried extracts of *S. trilobata* (1 kg of each), and 6.3, 5.9 and 6.1 mg g^{-1} of KA were found, showing the reproducibility of the extract preparation by spray drying in scale-up.

4. Conclusions

A selective, stability-indicative, precise, robust and accurate HPLC–UV method has been developed for quantifying KA in *S. trilobata* spray dried extract, being useful for future stability study of the medicinal plant and vegetable derivative and adapted for phytomedicines based on *S. trilobata*.

Acknowledgments

The authors are grateful to Laboratório Elofar (Florianópolis, SC, Brazil), Laboratório de Produção e Análise de Medicamentos da UNIVALI (UNIVALI-LAPAM; Itajaí, Brazil), Conselho Nacional de Ciência e Tecnologia (CNPq) and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC) for their financial support.

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