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# Determination of umckalin in commercial tincture and phytopreparations containing *Pelargonium sidoides* by HPLC: Comparison of sample preparation procedures

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ABSTRACT

Roots of *Pelargonium sidoides* D.C. are used for the production of phytomedicines. Current quality control of phytopreparations containing *P. sidoides* extracts has been made in terms of total phenolics content. In this work we describe the development and validation of an HPLC method for the analysis of *P. sidoides* tincture and commercial syrup phytopreparations using umckalin (7-hydroxy-5,6-dimethoxycoumarin) as chemical marker. Two sample preparation procedures, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were also developed and compared. The samples were analyzed by RP-HPLC and the two methods were then validated and compared. The repeatability of the two procedures showed coefficients of variation (CV) of 1.2% for SPE procedure, and 1.3% for LLE. Recovery for both methods was higher than 95.2%. The linearity showed correlation coefficients better than 0.999 for both methods. The detection and quantification limit were 0.0098 and 0.0298 $\mu g\,mL^{-1}$, respectively. The validated procedure was then used for the analysis of tincture and five batches of two commercial phytopreparations containing *P. sidoides* tincture.



## 1. Introduction

Roots of *Pelargonium sidoides* D.C. (Geraniaceae) are used for the treatment of acute and chronic infections of the respiratory tract and ear, nose and throat. Standardized extracts of the plant are now commercially available and widely used for the manufacture of phytopreparations usually in syrup form [1,2]. Phytochemical investigations have shown that the main constituents of *P. sidoides* are phenolic acids (e.g. gallic acid and its methyl ester), proanthocyanidins and several coumarins [1,3,4]. Among the coumarins the 7-hydroxy-5,6-dimethoxycoumarin (umckalin, Fig. 1) has only been found in this species [5]. The antimicrobial property of the extract and some coumarins has been established and umckalin was the most active against some pathogenic bacteria [3].

The standardization of phytomedicines is important for safety and efficacy reasons. The concentration of active compounds in plant material may vary geographically and seasonally. Therefore, before drug manufacture it is important to know the concentration of marker compounds in the raw material and adjust the composition in order to have a final product with defined concentration of those constituents [6].

Commercial phytopreparations formulated with a hydroethanolic extract of the plant available in Brazil are standardized in terms of total phenolics. The procedure has the same limitation as that indicated by the European Pharmacopoeia which describes the analysis of tannins for quality control of roots of *P. sidoides* and *P. reniforme* [7]. Considering that umckalin is present in high concentrations in the plant and that it has not been found in other species of the same family, like *P. reniforme*, this compound would be a good candidate as a chemical marker for products containing *P. sidoides*. Procedures for the analysis of coumarins in plant material have been described. For example, bergapten, imperatorin, cnidilin, osthole and isoimperatorin have been determined in *Angelica dahurica* by a validated HPLC-RP procedure [8]. For umckalin, however, the only report found on its determination in *P. sidoides* describes an HPLC-RP on silica-$C_{18}$ column but neither validation nor sample preparation optimization was described [9].

The objective of this work, therefore, was to evaluate and compare sample preparations methods for HPLC determination of umckalin in *P. sidoides* hydroalcoholic extracts and in commercial syrup formulations containing that extract.

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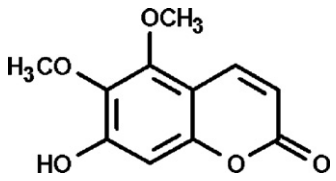


**Fig. 1.** Structure of umckalin (7-hydroxy-5,6-dimethoxycoumarin).

## 2. Experimental

### 2.1. Reagents and samples

All reagents were of an analytical grade. Acetonitrile was of HPLC grade (Merck, Darmstadt, Germany). HPLC grade water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). SPE cartridges of silica-$C_{18}$ were Chromabond® and Chromabond ec® (Macherey-Nagel). Nylon syringe filters (0.45 μm) were purchased from Millipore. Tincture of *P. sidoides* was supplied by Finzelberg (Berlin, Germany) and roots were purchased from African Bush (San Antonio, USA). Ten batches of two brands of syrup phytopreparations containing *P. sidoides* tincture were purchased from local suppliers.

### 2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Hewlett-Packard 1100 series (Agilent Technologies, USA) equipped with a quaternary pump, on line degasser, column heater, autosampler and diode-array detector (DAD). Data collection and analysis were performed using ChemStation software (Agilent Technologies). Separation in the final method was achieved on a Luna $C_{18}$ column 150 mm × 4.6 mm, 3 μm particle size (Phenomenex, Torrance, CA, USA) and, for comparison, on a Zorbax XDB $C_{18}$ column with the same dimensions and particle size (Agilent, Santa Clara, CA, USA). The elution was isocratic at 0.75 mL $min^{-1}$ with a mobile phase of acetonitrile–water (45:55, v/v). The column temperature was maintained at 30 °C. The injection volume was 10 μL with UV detection at 330 nm. NMR spectra were acquired with a DRX-400 instrument (Bruker, Rheinstetten, Germany), with sample prepared in $CDCl_3$ using TMS as internal standard. Mass spectra were recorded with an API 3000 spectrometer (Applied Biosystems, Foster City, USA), with an electrospray interface. A solution of umckalin (1 ppm) was prepared in 50% methanol in 5 mM ammonium formiate. Differential scanning calorimetry analysis was carried out with a DSC 260 instrument (TA Instruments, New Castle, USA) previously calibrated with metallic indium. The sample (*ca.* 3.0 mg) in a sealed aluminium capsule was heated under nitrogen (100 mL $min^{-1}$), at a rate of 10 °C $min^{-1}$ (40–300 °C).

### 2.3. Isolation, identification and purity determination of umckalin

*P. sidoides* tincture (1000 mL) was extracted with diethyl ether (3× 1000 mL), the solvent was evaporated and the residue (800 mg) was fractionated on a silica column (40 g). Elution was made with mixtures of hexane and diethyl ether of increasing polarity. The fractions containing umckalin were combined, the solvent evaporated and the residue recrystallized from diethyl ether. The identity of the isolated compound (20 mg) was confirmed by spectroscopic methods. Its NMR spectra were compatible with its chemical structure and with published data [5]. ESI-MS data confirmed its molecular mass.

The purity of umckalin was determined by HPLC–DAD analysis, and by differential scanning calorimetry in order to determine the content of crystallization solvent residue.

### 2.4. Sample preparation

*Liquid–liquid extraction* (*LLE*): the sample (20 mL of tincture) was extracted with diethyl ether (3× 20 mL). The solvent of the combined ether extracts was evaporated and the residues were reconstituted in MeOH (10 mL). Samples were then filtered and injected into the HPLC system.

*Solid-phase extraction* (*SPE*): cartridges of silica-$C_{18}$ (Chromabond® ec or Chromabond ec®) were conditioned with methanol (5 mL) and water (5 mL) and then samples (2 mL of tincture or 5 mL of syrup) were introduced. The cartridge was then washed with 20% aqueous acetonitrile–water (2 mL) before analyte elution with methanol (5 mL). After filtration samples were injected into the HPLC system.

### 2.5. Method validation

#### 2.5.1. Specificity

Specificity was determined by comparison of the UV spectra at upslope, apex and downslope portions of the peak of umckalin in HPLC–DAD chromatograms obtained from samples of pelargonium tincture and syrup. The same procedure was carried out with samples of tincture after acid (0.1 M HCl) and base (0.1 M NaOH) treatment of samples of pelargonium tincture [10]. Tincture (2 mL) was added to aqueous acid or base (2 mL) and the mixture was shaken for 1 h at 25 °C. The mixture was neutralized and then analyzed.

#### 2.5.2. Linearity

Linearity was evaluated for analytical curves using three different procedures. In the first procedure standard umckalin solutions in the range of 0.2–121.2 μg $mL^{-1}$ were used. Three sets of five different calibration solutions were injected into the chromatographic system in triplicate, and in 3 different days. The 15-point analytical curve was plotted and statistically evaluated.

Linearity was also evaluated for tincture samples, prepared using LLE procedure, with concentrations of 80, 90, 100, 110 and 120% of theoretical value. Those at 80–100% were prepared by dilution with 11% ethanol in water. Samples with 110 and 120% of theoretical value were prepared by addition of a sufficient volume of an umckalin solution in the same solvent in order to achieve final concentrations of 3.0 and 6.0 μg $mL^{-1}$, respectively.

For syrup 5 samples were prepared by SPE method and diluted or spiked with umckalin, as described above, in order to achieve final concentrations equivalent to 80–120% of theoretical value.

#### 2.5.3. Accuracy

The true umckalin concentration in tincture syrup was determined using a procedure known as integrated calibration method, which combines regular calibration plot with that prepared by the method of standard addition [11]. Solutions were prepared with umckalin in solvent and with samples to which increased amounts of standard were added and then prepared by LLE and SPE procedures. The umckalin concentration in the sample was determined by extrapolating the value of the linear coefficient of the equation for spiked sample solutions into that of the standard equation, and the concentration was corrected using a factor calculated for dilution and sample size. For syrup samples accuracy was determined by addition of tincture with umckalin concentration previously determined by the standard addition procedure to placebo.

From the true concentration values obtained for tincture and syrup samples with 80, 100 and 120% of theoretical values were prepared in triplicate, under the same conditions used for linearity tests. After analysis recovery was calculated.

**Table 1**
Parameters used for robustness evaluation of the chromatographic method.

| Parameter | Standard condition | Altered conditions | |
|---|---|---|---|
| | | Inferior | Superior |
| Column | Phenomenex Luna $C_{18}$ (150 mm × 4.6 mm × 3 μm) | Agilent Zorbax XDB $C_{18}$ (150 mm × 4.6 mm × 3 μm) | |
| % Acetonitrile in mobile phase | 45 | 43 | 47 |
| Flow (mL min$^{-1}$) | 0.75 | 0.70 | 0.80 |
| Column temperature (°C) | 30 | 28 | 32 |

*2.5.4. Precision*

Within-day precision (repeatability) was evaluated by repeated analyses of tincture samples, at 100% of the test concentration ($n = 6$), and between-day precision (reproducibility) was evaluated by two analysts in 2 consecutive days ($n = 6$). The concentration of umckalin was determined and the relative standard deviation (RSD) calculated and compared.

*2.5.5. Detection and quantitation limits*

Detection and quantitation limits (LOD and LOQ, respectively) were evaluated for solutions of umckalin ($n = 5$) of decreasing concentrations (1.5–0.081 μg mL$^{-1}$). They were calculated according to the equations LOD = 3.3$\delta$/S, LOQ = 10$\delta$/S, where $\delta$ is the standard deviation of responses and $S$ is the slope of the analytical curve [12].

*2.6. Robustness*

Robustness was determined for the chromatographic procedure. The parameters evaluated and standard and altered conditions are summarized in Table 1. All experiments were carried out in triplicate and compared.

The stability of standard solutions was determined using five solutions (12.1–121 μg mL$^{-1}$) which were analyzed immediately after preparation and after 30, 60, 90 and 180 days of storage at −20 °C. The respective responses were plotted and compared. The stability of samples was determined by analysis immediately after preparation and after 24 h of storage at 20 °C.

## 3. Results and discussion

*3.1. Umckalin isolation and purity determination*

Umckalin is not commercially available. Therefore, its isolation, characterization and purity determination were essential for this work. Tincture of *P. sidoides* was used as the source and the isolation procedure involved an initial extraction with diethyl ether followed by chromatographic fractionation on silica. Spectroscopic analysis (NMR and ESI-MS) of the isolated compound and comparison with the literature data confirmed its identity [5,9]. The purity was determined by HPLC and DSC analysis. The chromatogram showed a small percentage of an impurity (0.61%), identified as scopoletin by comparison with the authentic sample. Calorimetric analysis indicated 98.71% purity for umckalin due to a small loss of water and crystallization solvent.

*3.2. Tincture sample preparation*

Two methods for sample preparation, LLE and SPE, were developed and compared. For LLE ether was selected due to its low boiling point and efficiency. Parameters studied were solvent volume, number of extractions and agitation time. The conditions selected (20 mL of tincture extracted with 3× 20 mL of diethyl ether) provided a recovery close to 100% since no umckalin was detected in the extracted tincture after direct injection into the HPLC system.

For SPE, two types of silica-$C_{18}$ cartridges were tested, Chromabond® or Chromabond ec®, the latter end-capped. Both showed similar retention and capacity for umckalin. The saturation volume of tincture was evaluated by consecutive additions of 0.5 mL aliquots of sample and analysis of eluate. The cartridge capacity was 2.5 mL of tincture which contained 75 μg of umckalin. The volume of 2 mL was then established for practical reasons. Cleaning was attempted with aqueous acetonitrile (5, 10, 20 and 30%). Concentrations up to 10% required volumes higher than 5 mL whereas 1 mL of 30% acetonitrile induced breakthrough of umckalin. Therefore, cleaning was optimized with 2 mL of 20% acetonitrile. Final recovery step was evaluated with different volumes of acetonitrile or methanol. The latter was more efficient for removal of umckalin from cartridge and 5 mL of the solvent was sufficient for recovery close to 100%. Both methods were efficient in removing most of the polar components of the samples (Fig. 2).

*3.3. Syrup sample preparation*

Preparation of syrup samples was made by SPE with the same conditions used with the tincture. ChromaBond® $C_{18}$ ec cartridge showed a capacity of 30 μg of umckalin (5 mL of sample). This lower capacity, compared with that for tincture, may be explained by the presence of a high content of sucrose reducing the efficiency of the

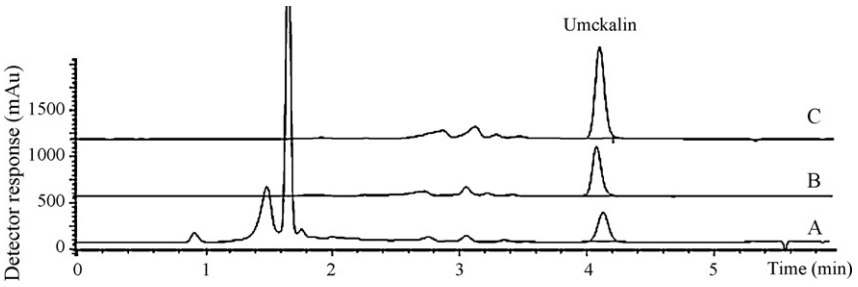


**Fig. 2.** HPLC analysis of *P. sidoides* tincture before (A) and after preparation by LLE (B) and SPE (C).

**Table 2**
Linearity for standard and samples of tincture and syrup prepared by LLE and SPE ($n = 3$).

| | Method | Sample preparation | Function | $R^2$ | Range ($\mu g\,mL^{-1}$) |
|---|---|---|---|---|---|
| Standard | | | $y = 45.812x + 3.9731$ | 0.9995 | 0.2–121.2 |
| Tincture | Standard addition | LLE | $y = 47.849x - 104.8$ | 0.9982 | 48.11–72.17 |
| | Standard addition | SPE | $y = 42.894x + 34.635$ | 0.9989 | 9.62–14.43 |
| Syrup | Standard addition | SPE | $y = 51.395x - 32.574$ | 0.9979 | 4.81–7.22 |

stationary phase. Cleaning and elution steps were the same used for tincture.

### 3.4. Chromatographic analysis

Optimization of HPLC analysis was attempted with a few $C_{18}$ columns and different mobile phase compositions. Although separation was possible with 5 μm particle size columns we decided to optimize the separation using a Luna $C_{18}$ column (150 mm × 4.6 mm) with 3 μm granulometry for shorter analysis time. Using acetonitrile–water (45:55, v/v) as mobile phase, pumped at 0.75 mL $min^{-1}$, analysis could be completed in less than 5 min and with a good separation of analyte peak as can be seen in the chromatogram of tincture before sample preparation (Fig. 2A).

### 3.5. Method validation

The method was validated according to current guidelines of ICH [13] and ANVISA (Brazil). Specificity and selectivity were evaluated by the spectral purity of umckalin peak by means of the DAD. Samples of tincture were also subjected to acid or basic hydrolysis in order to generate potential degradation products [10]. The peak purity was unchanged even after these stress conditions indicating no co-eluting compounds.

#### 3.5.1. Linearity

Linearity was determined for standard umckalin solutions and for the two matrices, tincture and syrup (Table 2). Initially, an analytical curve was constructed in the usual way using standard umckalin solutions with concentrations in the 0.2–121.2 $\mu g\,mL^{-1}$ range. The results showed linearity in the range studied and the residuals plot (not shown) showed random (normal) distribution indicating no bias or outliers.

This method of linearity determination, although useful for showing proportionality between standard concentration and detector response, may not be adequate for applications on complex mixtures like plant extracts or phytopreparations. Interferents may influence detector response at varying degrees depending on sample concentration. Likewise, recovery may also vary according to analyte concentration. Therefore, alternative procedures which take sample matrix into consideration, may be more appropriate for complex samples.

An alternative method for linearity determination was executed with samples of tincture and syrup prepared as described above. Dilution or spiking was used in order to obtain a concentration range of 80–120% of theoretical value. The results (Table 2) showed that linearity was slightly poorer than that obtained with standard solutions. However, they were within recommended parameters indicating that, for those matrixes, linearity was maintained in the samples for the analyte range chosen.

**Table 3**
Recovery of umckalin from tincture according to sample preparation method and concentration level ($n = 3$).

| Method | 80% (27 $\mu g\,mL^{-1}$) | 100% (30 $\mu g\,mL^{-1}$) | 120% (33 $\mu g\,mL^{-1}$) |
|---|---|---|---|
| LLE | 98.09 ± 0.39 | 98.90 ± 0.94 | 95.09 ± 0.53 |
| SPE | 98.15 ± 1.10 | 95.12 ± 1.12 | 97.46 ± 0.30 |

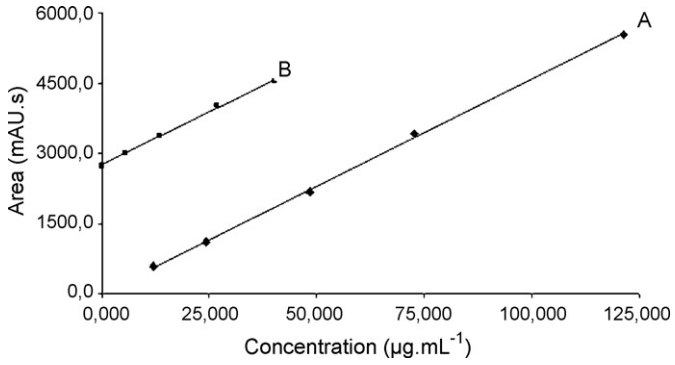


**Fig. 3.** Calibration curves prepared with solutions of umckalin standard (A) and with tincture samples spiked with increasing amounts of umckalin (B) followed by preparation by LLE ($n = 3$).

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from calibration data as described in Section 2.5.5 [12]. The results found were 0.0098 and 0.0298 $\mu g\,mL^{-1}$, respectively.

#### 3.5.2. Accuracy

For complex mixtures such as plant extracts it is not possible to have analyte free matrixes. In these cases the method of standard addition is more indicated for the preparation of calibration curves to be used for accuracy evaluation. Therefore, solutions were prepared with umckalin in solvent and with samples to which increased amounts of standard were added and then prepared by LLE and SPE procedures (Table 3). The respective curves are shown in Figs. 3 and 4. Extrapolation of the response of concentration zero for the standard addition line (B) to the standard line (A), followed by correction for dilution, produced the concentrations of 30.02 and 30.12 $\mu g\,mL^{-1}$ for LLE and SPE sample preparation procedures, respectively.

The matrix effect could be estimated for both sample preparation procedures. The slope ratio (SR) was calculated by $SR = S_1/S_2$, where $S_1$ and $S_2$ are the slopes for the plots of spiked samples and standards, respectively. The results found for LLE and SPE, 0.98 and 0.99 respectively, indicate similar but small influence of matrix effect.

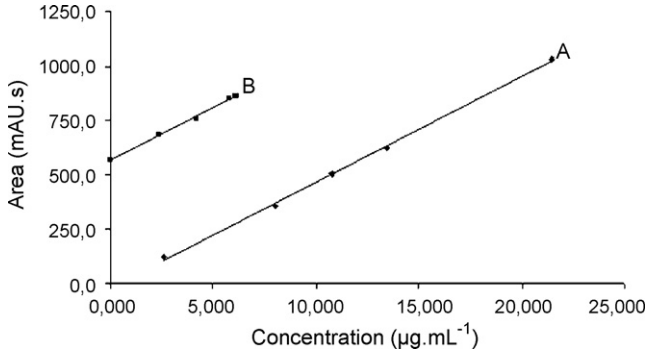


**Fig. 4.** Calibration curves prepared with solutions of umckalin standard and with tincture samples spiked with increasing amounts of umckalin followed by preparation by SPE ($n = 3$).

**Table 4**
Stability of samples of tincture and syrup prepared by liquid–liquid extraction (LLE) and solid-phase extraction (SPE) stored at 20 °C. Umckalin peak areas are the means of 6 replicates.

| Sample | Tincture (LLE) | | Tincture (SPE) | | Syrup (SPE) | |
|---|---|---|---|---|---|---|
| Time (h) | 0 | 24 | 0 | 24 | 0 | 24 |
| Mean | 2779.6 | 2785.8 | 551.5 | 550.2 | 278.1 | 277.0 |
| SD | 37.8 | 31.9 | 6.6 | 9.6 | 2.7 | 3.6 |
| RSD (%) | 1.3 | 1.1 | 1.2 | 1.7 | 1.0 | 1.3 |

After determination of the umckalin concentration the accuracy of the method was investigated by recovery experiments at three concentration levels. The results, summarized in Table 3, show that recoveries were higher than 95% with very low relative standard deviations.

### 3.5.3. Precision

In the intra-day precision analyses ($n = 6$), the mean content of umckalin was 30.31% (RSD = 1.29%) for LLE method and 28.99% (RSD = 1.17%) for SPE method. The values found in the inter-day precision ($n = 12$) were 29.66% (RSD = 2.91%) for LLE method and 29.24% (RSD = 1.60%).

### 3.6. Robustness

The results for method robustness showed that only variations in column temperature did not influence analytical results. Changes in all other factors – column brand and mobile phase flow and composition – significantly altered the quantifications with small changes in retention time and peak shapes which affected integration.

Stability of samples and standard solutions were evaluated. The results showed that the samples ready for analysis were stable for at least 24 h after preparation (Table 4). The stability for standards was determined by injecting calibration solutions into the chromatographic system at 30-day intervals. The results (not shown) resulted in five superimposable lines indicating excellent stability of calibration solutions under storage conditions (−20 °C).

### 3.7. Commercial samples analysis

The validated method was then used for the analysis of five batches of two commercial phytopreparations containing *P. sidoides* tincture. The results for both products were similar in umckalin concentration ($21.58 \pm 1.96$ and $21.04 \pm 1.05\ \mu g\ mL^{-1}$). Comparison with label data however, was not possible because those products are standardized in terms of total phenolics.

## 4. Conclusion

Our results provide a fully validated HPLC method for quality control of plant extracts and phytopharmaceuticals containing *P. sidoides*, using umckalin as chemical marker. The method is more appropriate than the current practice of standardization in terms of total phenolics or of tanins as described in European Pharmacopoeia [7]. Both sample preparation procedures, LLE and SPE, gave similar results the former being more economical and adequate for a smaller number of samples whereas the latter is indicated for a larger number of samples and automation. Validation of the method according to ICH and ANVISA (Brazil) showed that the method was in accordance with current guidelines. The method was robust for small variation in column temperature but not for the other parameters like column brand, mobile phase composition and flow rate. In these cases recalibration is necessary.

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