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Simultaneous Determination of Coumarin, *O*-Coumaric Acid, Dihydrocoumarin and Syringaldehyde in Guaco Extracts and Pharmaceutical Preparations by HPLC-DAD

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Abstract

In this study a new HPLC-DAD method for simultaneous determination of coumarin, *o*-coumaric acid, dihydrocoumarin and syringaldehyde in guaco extracts and pharmaceutical preparations without sample pre-treatment has been developed. The chromatographic separation was carried out on a XBridge C18 (150 x 4.6mm, 5µm) column maintained at room temperature. The mobile phase consisted of water/methanol/acetonitrile/formic acid (65:30:5:1, *v/v/v/v*) eluted at a flow rate of 1.0 mL min^{-1} in an isocratic system. The validation procedures showed excellent selectivity and linearity over a range of 1.0 to 200 µg mL^{-1} for all compounds ($r > 0.999$). The range of recovery was 97.9 to 101.8% with a RSD < 5% for intra-day and inter-day precision. The robustness study indicated that flow rate was the only critical factor. Sample analyses demonstrated a lack of standardization in the amounts of the main guaco metabolites among the evaluated samples. The new method is presented as an alternative for the quality control of guaco extracts and pharmaceutical preparations.

Keywords: Guaco; HPLC; Coumarin; o-Coumaric acid; Dihydrocoumarin; Syringaldehyde

Introduction

Mikania glomerata Sprengel and *M. laevigata* Schultz Bip. ex Baker, commonly known as guaco, are medicinal species used for the treatment of several inflammatory and allergic conditions, particularly in the respiratory system due to their bronchodilator properties [1-4]. Because of the therapeutic effects attributed to these species, the preparations syrup and oral solution are widely used by the South American population, and have been distributed for free government phytotherapy programmes [5,6].

The pharmacological effects of guaco are attributed mainly to the presence of coumarin (1,2-benzopyrone), however other metabolites have demonstrated synergism to produce the desirable pharmacological effects. Studies that evaluated isolated markers in the mice allergic pneumonitis model have demonstrated that coumarin and *o*-coumaric acid are part of the phytocomplex that is responsible for the therapeutic activity of the guaco species [2]. Besides these, dihydrocoumarin [7] and syringaldehyde [8], recently described as the major compounds in hydroalcoholic extracts, revealed the antioxidant, immunologic and anti-inflammatory properties [9-14]. Therefore the presence of these metabolites is directly related to the guaco benefits and its monitoring considered an important tool for the extracts characterization, quality control and therapeutic effects warranty.

Despite the pharmacological relevance of these substances, in the literature only one method was described for the simultaneous determination of three of these metabolites in guaco extracts [15]. However this method does not apply to the pharmaceutical preparations due to the complex matrices composed mainly for honey, preservatives, sweetners and in some cases other plant associations. Consequently, for pharmaceutical preparations, mostly of developed methods require several extraction processes [16-18] and therefore are laborious and require high time and chemical-consuming. In other case, using UV analysis no extraction process was applied [19], however no assurance can be asserted specially regarding the selectivity, once the method was not validated and preservatives and another syrup constitutents can absorb in the same wavelength chosen [16].

Techniques such high performance liquid chromatography (HPLC) [17] and gas chromatography (GC) [18] have also been used for the guaco phytomedicines quality control. However, despite the high potencial of selectivity of these techniques, all avaliable methods monitor only coumarin as marker, and no other metabolite related to the guaco therapeutic effects. To meet these requirements, recently our research group published LC-MS/MS methods for the determination of the major guaco metabolites in extracts and preparations [20]. Nevertheless, the development of alternative techniques such as HPLC-DAD becomes important, especially because the most of research and routine analysis laboratories use this system for qualitative and quantitative assay.

This work deals the development and validation of a new method for simultaneous determination of coumarin, *o*-coumaric acid, dihydrocoumarin and syringaldehyde directly in guaco extracts and pharmaceutical preparations (syrup and oral solution) without the need for sample pre-treatment. Therefore for those methods described in the literature, it may be a simple alternative to quantify the main

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guaco metabolites in complex matrices, and thus it can be used for the quality control in all drug-processing stages.

Materials and Methods

Standards, chemicals and reagents

All reagents were of analytical grade, and the HPLC solvents were of chromatography purity. Methanol, acetonitrile and formic acid 88% were purchased from J. T. Baker Chemicals B. V. (Deventer, Netherlands). Samples of guaco syrup and oral solution (n=12) were obtained from local markets (Curitiba, Brazil). The avaliable commercial extracts (aqueous and hydroalcoholic tincture, n=2) were kindly donated by the Herbarium Botanical Laboratory. Ultrapure water was obtained using a Milli-Q purification system from Millipore Corporation (Bedford, USA). The columns (150 x 4.6 mm i.d., 5µm particle size) Spherisorb ODS2 and XBridge were purchased from Waters Corporation and Zorbax Eclipse XDB from Agilent Technologies. Standards of 1,2-benzopyrone (99.0%), dihydrocoumarin (99.0%), *o*-coumaric acid (97.0%) and syringaldehyde (98.0%) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The structures of each chemical are shown in Figure 1.

Standard solutions

Stock solutions of 1,2-benzopyrone, *o*-coumaric acid, syringaldehyde and dihydrocoumarin were prepared separately in methanol at a concentration of 1 mg mL^{-1}. All stock solutions were stored under refrigeration at 4°C. Working standard solutions were freshly prepared by mixing the stock solutions in water/methanol/acetonitrile (65:30:5, *v/v/v*) to reach a concentration of 200 µg mL^{-1} of each compound. From these solutions, other working standard solutions were prepared through new dilutions as needed. All working standard solutions were filtered through a polyvinylidene fluoride (PVDF) syringe filter (11 mm, 0.45 µm, Millipore Millex, Billerica, USA) before injection into the HPLC system.

Sample preparation

The samples of guaco syrup and oral solution were diluted according to the coumarin content declared in the leaflet. Thus, all samples were diluted 1:1 *v/v,* in water/methanol/acetonitrile (65:30:5, *v/v/v*), except one sample, that had a declared coumarin content ten times greater than the other samples. For this sample, two steps of dilutions were necessary, 1:10 *v/v* and then 1:1 *v/v*. Guaco extracts were directly diluted in a diluent solution composed of ethanol/water (70:30 *v/v*). Thus, aqueous concentrated extract was diluted 1:50 *v/v* and hydroalcoholic tincture 1:20 *v/v*. All solutions were filtered through a 0.45 µm PVDF syringe filter before injection.

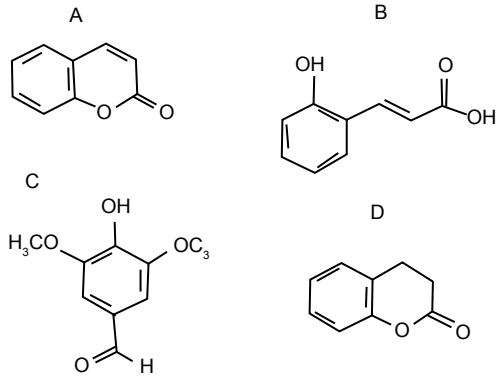

Figure 1: Chemical structures of coumarin (A), *o*-coumaric acid (B), syringaldehyde (C) and dihydrocoumarin (D).

Instrumentation and chromatographic conditions

The analyses were carried out on an Agilent 1100 LC system (Wilmington, USA), that consisted of a G1311A quaternary pump, G1379A degasser, G1329A automatic injector, G1315B photo diode array detector and an LC workstation equipped with Chemistation A. 10.02 software for data collection and acquisition. Chromatographic separations were carried out on a Zorbax Eclipse XDB C_{18} (Agilent Technologies Inc., California, USA) 150 x 4.6 mm i.d., 5 µm column connected to a Zorbax Eclipse XDB C_{18} (12.5 x 4.6 mm i.d., 5-µm) pre-column, maintained at room temperature. The mobile phase consisted of water/methanol/acetonitrile/formic acid (65:30:5:1, *v/v/v/v* - pH ~2.5) at a flow rate of 1.0 mL min^{-1} in isocratic elution mode. Mobile phase were filtered through a 0.45 µm PTFE membrane (Millipore, Molsheim, France). The detection wavelength was set at 274 nm and the injection volume of the sample was 20 µL.

Method validation

The validation of analytical procedures was executed under ICH guidelines [21]. According this guideline, the key parameters to ensure the acceptability of the performance of an analytical method are selectivity, linearity, limits of detection and quantification, precision, accuracy, and robustness. All validation procedures were applied only in phytomedicines (guaco syrup and oral solution), once the extracts are part of these matrices.

Selectivity

To confirm the selectivity two methods were applied for both samples (guaco syrup and oral solution).

The method of addition of standard consists of the comparison of one analytical curve obtained with standard solutions, with another analytical curve obtained with sample spiked with analytes. In the proposed method, working standard solutions of coumarin, *o*-coumaric acid, syringaldehyde and dihydrocoumarin were injected in triplicate at three concentration levels of 2.5, 25 and 100 µg mL^{-1} of each compound. The data was treated by linear regression and an analytical curve was obtained. The same concentration levels were used to spike the oral solution and the syrup. These samples were injected in triplicate and the same mathematical treatment was used to obtain the analytical curve. The slopes obtained from these analytical curves were then compared.

The other method used to determine the selectivity was through the analysis by photo diode array detector (DAD). This method compared the bands of ultraviolet absorption of the chromatographic peaks of the standards with those obtained in the samples. If there is no displacement of the absorption bands, the method can be considered selective.

Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were estimated from the signal-to-noise ratio [21]. Thus, to conduct this study, working standard solutions at 10.00 µg mL^{-1} for each compound were diluted in water/methanol/acetonitrile (65:30:5, *v/v/v*) until the smallest detectable peaks were reached. The

LOD was estimated at a signal-to-noise ratio of 3:1, and the LOQ was estimated as the peak at a signal-to-noise ratio of at least 10:1, until the obtention of desirable accuracy and precision.

Linearity and linear range

The linearity was studied through an external standardization method by testing mixtures of the working standard solutions at eight concentration levels of each compound. Due to the expectative of a wide variation in the metabolites contents among the samples, the calibration curves covered an ample range of concentration ranged from 1 to 200 μg mL^{-1} for each compound. The samples were diluted in water/methanol/acetonitrile (65:30:5, *v/v/v*) and each concentration level was injected in triplicate. Calibration curves were constructed by plotting the ratio of the mean peak areas versus concentration. The linearity was assessed by linear regression analysis.

Accuracy and precision

The precision was evaluated using measurements of the repeatability (intra-day) and intermediate precision (inter-day). The repeatability was investigated using working standard solutions at concentration levels of 2.5, 25 and 100 μg mL^{-1} for all compounds. The samples were injected in triplicate, and the results were expressed as the Relative Standard Deviation of measurements (RSD%). Intermediate precision was determined by a second analyst at the same concentration levels of repeatability after four consecutive days.

The accuracy of the method was measured in triplicate through a recovery assay. To perform this assay, working standard solutions (standard concentrations) were prepared at concentration levels of 2.5, 25 and 100 μg mL^{-1} for each compound. Then, a sample of guaco syrup was diluted 1:10 *v/v* in water/methanol/acetonitrile (65:30:5, *v/v/v*) and injected into the HPLC system. After the injections, the diluted samples were spiked with working standard solutions in the same concentration levels of the standard concentrations. The amount of analytes recovered was calculated by subtracting the values of the analytes found in spiked samples from those obtained in samples without fortification. The accuracy was expressed as a percentage of the amount recovered compared to standard concentrations.

Robustness

The robustness of the proposed method was tested by small variations in the proportions of methanol (29-31%), acetonitrile (4.9-5.1%) and formic acid (0.9-1.1%) in the mobile phase compositon. The flow rate (0.98 to 1.02 mL min^{-1}) and the column temperature (23 to 27°C) were also varied. The analyses were accomplished using a six replicates of working standard solution at 25 μg mL^{-1} of each compound. Statistical analyses (RSD% and Student t-test) were performed to compare the behavior of the data after the deliberate changes.

Results and Discussion

Chromatographic optimization

In order to achieve simultaneous determination of coumarin, dihydrocoumarin, *o*-coumaric acid and syringaldehyde in guaco extracts, syrup and oral solution without sample pre-treatment, different chromatographic conditions and brands of C_{18} columns were investigated. The choice of the column was based on peak shape and resolution.

In an initial experiment, a Zorbax XDB C18 (150 x 4.6 mm i.d., 5 μm particle size) column maintained at room temperature was selected for separations. The mobile phase composition was also tested across several proportions of water and methanol (80:20 to 60:40, *v/v*) at a flow rate of 0.7 mL min^{-1}. These conditions, however, displayed poor peak symmetry for coumarins (1,2-benzopyrone, dihydrocoumarin) with a tailing factor higher than 1.5. The addition of 1% formic acid into the mobile phase (pH ~2.5) show a significant reduction of the tailing factor to values lower than 1.1. As a result, better separation was achieved using a mobile phase composed of water/methanol (70:30, *v/v*) with 1% formic acid, but a much higher retention time for dihydrocoumarin (t_r > 30 min) was observed. Therefore, the system was not considered adequate for use, being necessary to use an organic solvent capable to decrease the run time without produce extreme changes in the mobile phase polarity. Thus small amounts of acetonitrile (2-7%) were incorporated in the mobile phase, with an equal proportion of water removed. Finally, the mobile phase composed of water/methanol/acetonitrile/formic acid (65:30:5:1, *v/v/v/v*) was found as the optimal, since the retention time of dihydrocoumarin has decreased considerably (t_r = 20 min), and the separation of the analytes of interest, as well as the sweeteners, preservatives and other components present in the samples was improved.

Different temperatures (25 to 35°C) and flow rates (0.7 to 1.5 mL min^{-1}) were also evaluated. The investigation showed that temperatures higher than 27°C caused significant reduction of the resolution between 1,2-benzopirone, methylparabene and *o*-coumaric acid. In addition, the increases in the flow rate has decreased the run time and also improved peak symmetry without losing resolution among the compounds. However, flow rates greater than 1.0 mL min^{-1} did not demonstrate significantly cost/benefit ratio.

The system was tested in different C_{18} columns (see standards, chemicals and reagents section) and the Spherisorb ODS2 did not provide efficience for separation. XBridge presented higher separation, but should be used with caution because low resolution (1.3) was observed between coumarin and methylparabene peaks.

Finally the best conditions were achieved using a Zorbax Eclipse XDB C_{18} (150 x 4.6 mm i.d., 5 μm particle size) column, maintained at room temperature, using a mobile phase consisting of water/methanol/acetonitrile/formic acid (65:30:5:1, *v/v/v/v*) at a flow rate of 1.0 mL min^{-1} in isocratic elution mode. Under these conditions, the analyte peaks were well resolved and the chromatographic system presented excellent values of USP tailing (*T*), plate number (*N*), retention factor (k´) and resolution (R_s), as described in Table 1.

The developed method presents several advantages in comparison with others described in the literature. For example, mostly of published

Compounds	Parameters (Mean=6)				
	t_r (min)	*k´*	R_s	*T*	*N*
Syringaldehyde	4.526	3.11	1.77	1.11	8287
Coumarin	8.624	6.84	3.02	1.05	13453
o-coumaric acid	10.572	8.61	2.93	1.05	10852
Dihydrocoumarin	20.223	17.38	17.13	1.04	12644

Table 1: Retention time (t_r), retention factor (k'), resolution (Rs), tailing factor (*T*) and number of theoretical plates (N) from the system suitability study.

methods require several extraction processes [16-18] and therefore are laborious and require high time and chemical-consuming. In other cases no procedures of validation were applied [16,19] or the methods are especific for the determination of only one biomarker [17,18]. In the present work, the new method was totally validated and sucessfully applied for the simultaneous determination of the main guaco metabolites in extracts and preparations.

Muceneeki and co-workers [15] published a method that uses HPLC-DAD technique for the determination of three guaco metabolites in aqueous and hydroalcoholic extracts. However this method does not apply to the pharmaceutical preparations due to the complex matrices composed mainly for honey, preservatives, sweetners and in some cases other plant associations. Therefore it is possible to conclude that the present method has high selectivity because more metabolites were determined in both, extracts and preparations without require any sample pre-treatment. In addition, best separation was reached using lower flow rate, reducing significantly the environmental waste production.

Method validation

Selectivity: Due to the complexity of the matrices, two different methods were required to determine the selectivity. By the DAD detector, high overlapping UV absorption profiles was observed among the standards and the sample compounds (Figure 2). Therefore high peak purity was confirmed for both matrices (syrup and oral solution). Through the addition of standard method, the slopes comparison from both curves (standard solutions and spiked samples), shows no significant variation for all compounds (Table 2). Thus no additional interferences were observed in the same retention time of the analytes

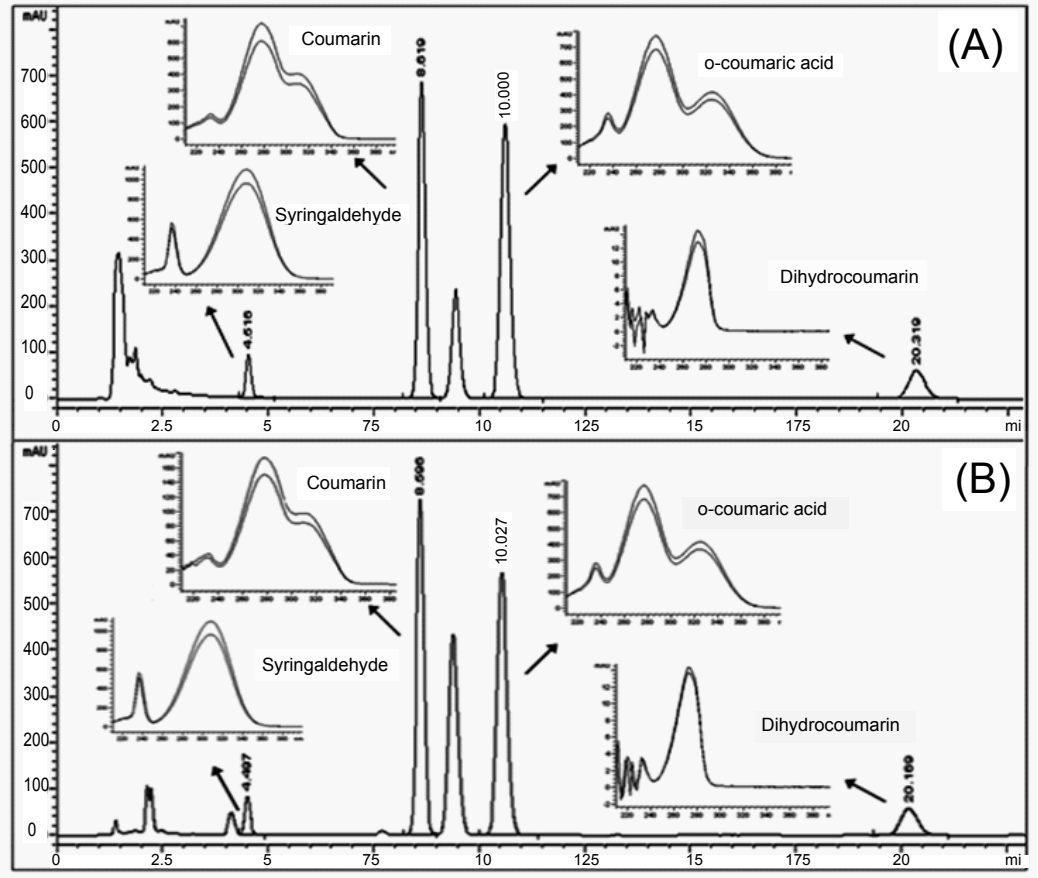

Figure 2: Chromatograms from selectivity study in (A) syrup matrix and (B) oral solution matrix.

Compounds	Slope					
	Sucrose syrup			Oral Solution		
	Standards	Spiked samples	RSD%	Standards	Spiked samples	RSD%
Coumarin	83.384	82.582	0.68	83.384	83.597	0.18
o-coumaric acid	94.630	96.996	1.75	94.630	94.812	0.14
Syringaldehyde	7.320	7.424	1.00	7.320	7.297	0.22
Dihydrocoumarin	15.987	15.244	3.36	15.987	16.063	0.34

Table 2: Comparative slope of standards and spiked samples in different matrices for selectivity study.

of interest. Therefore the new method was considered selective and appropriated for the determination of the main guaco metabolites in complex matrices such syrup and oral solution.

Limits of detection and quantification: The high sensitivity of the developed method was demonstrated by the low limits of detection estimated at 0.020 μg mL^{-1} for coumarin, 0.039 μg mL^{-1} for *o*-coumaric acid, 0.156 μg mL^{-1} for syringaldehyde and 0.313 μg mL^{-1} for dihydrocoumarin. The limits of quantification were 0.157 μg mL^{-1} for coumarin and *o*-coumaric acid, 0.625 μg mL^{-1} for syringaldehyde, and 1.0 μg mL^{-1} for dihydrocoumarin. The lowest concentration used with acceptable precision and accuracy to generate the inferior limit of the calibration curves was 1.0 μg mL^{-1} for all compounds.

Linearity and linear range: The calibration curves for coumarin, *o*-coumaric acid, syringaldehyde and dihydrocoumarin were considered linear for the evaluated concentrations with correlation coefficients (r) > 0.999. The linear regression equations and correlation coefficients (r) were as follow: coumarin, y = 76.809x – 43.919 (r = 0.9996); *o*-coumaric acid, y = 93.609x – 77137 (r = 0.9993); syringaldehyde, y = 7.2339x – 5.3093 (r = 0.9994); dihydrocoumarin, y = 15.448x + 2.0883 (r = 0.9999). These results guarantee a reliable response independent of the utilized concentrations.

Precision and accuracy: The results presented in Table 3 showed that the new method was precise for all compounds with RSD variations from 0.36 to 4.73% for intra-day, and 0.21 to 4.56% for inter-day analysis. Additionally, the method showed notable accuracy with satisfactory recoveries for all compounds at a range of 97.86 to 101.79%.

Robustness: In terms of selectivity, the robustness studies indicated that *o*-coumaric acid was the most sensitive metabolite (Table 4) when

Compounds	Accuracy			Precision	
	Standard concentration (μg mL^{-1})	Amount recovered (μg mL^{-1})	Main recovery (%)	Intra-day (RSD%)	Inter-day (RSD%)
Coumarin	2.5047	2.523	100.72	0.3625	0.9754
	25.047	25.040	99.97	0.7470	2.0431
	100.188	98.438	98.25	0.3558	3.5340
o-coumaric acid	2.488	2.524	101.45	1.2844	2.5572
	24.880	25.326	101.79	1.4173	3.4059
	99.522	99.735	100.21	2.1339	3.3561
Syringaldehyde	2.4849	2.467	99.27	4.7263	0.5547
	24.849	24.979	100.52	1.4382	2.5839
	99.396	99.620	100.22	2.2997	2.7535
Dihydrocoumarin	2.4948	2.441	97.86	1,4121	4.5559
	24.948	24.913	99.86	3.3594	0.2098
	99.792	99.917	100.13	4.3471	1.1586

Table 3: Precision and accuracy of coumarin, *o*-coumaric acid, dihydrocoumarin and syringaldehyde.

Robustness parameter	Coumarin				*o*-coumaric acid				Dihydrocoumarin				Syringaldehyde		
	T*f*	RSD%	R*s*	RSD%	T*f*	RSD%	R*s*	RSD%	T*f*	RSD%	R*s*	RSD%	T*f*	RSD%	R*s*
Methanol															
29%	1.03		16.3		1.04		5.80		1.03		17.4		1.13		-
30%*	1.04	0.36	16.5	1.65	1.04	0.14	5.38	5.45	1.03	0.17	17.1	1.54	1.14	0.49	-
31%	1.04		16.9		1.04		5.23		1.03		16.9		1.14		-
Acetonitrile															
4.9%	1.04		16.7		1.05		5.64		1.04		17.1		1.05		-
5%*	1.05	0.72	16.4	1.20	1.05	0.24	5.31	4.96	1.04	0.80	16.8	0.93	1.08	1.87	-
5.1%	1.06		16.4		1.05		5.11		1.04		16.9		1.09		-
Formic Acid															
0.9%	1.04		16.4		1.04		5.56		1.03		16.8		1.04		-
1%*	1.04	0.20	16.4	0.32	1.04	0.40	5.35	2.24	1.04	0.45	16.9	0.33	1.08	1.73	-
1.1%	1.04		16.3		1.03		5.35		1.04		16.9		1.06		-
Temperature															
23 °C	1.04		13.7		1.04		4.68		1.04		14.0		1.13		-
25 °C*	1.05	0.72	13.7	0.83	1.04	0.33	4.32	9.52	1.04	0.33	14.2	2.68	1.12	0.59	-
27 °C	1.06		13.9		1.04		3.88		1.03		14.7		1.12		-
Flow Rate															
0.98 mL min^{-1}	1.13		13.5		1.10		4.25		1.16		13.5		1.16		-
1.0 mL min^{-1}	1.14	0.45	13.4	0.19	1.10	0.51	4.26	0.47	1.17	0.25	13.4	0.19	1.17	0.25	-
1.02 mL min^{-1}	1.15		13.5		1.11		4.29		1.17		13.5		1.17		-

Data: *Optimized conditions; T*f*, tailing factor; R*s*, resolution

Table 4: Data of tailing factor and resolution in different conditions for robustness study.

Robustness parameter	Coumarin**			*o*-coumaric acid**			Dihydrocoumarin**			Syringaldehyde**		
	Recovery%	RSD%	T test (*p* value)	Recovery%	RSD%	T test (*p* value)	Recovery %	RSD%	T test (*p* value)	Recovery %	RSD%	T test (*p* value)
Methanol	Confidence interval = 99.26 to 100.74%			99.22 to 100.78%			99.50 to 100.35%			98.78 to 101.22%		
29%	99.97	0.09	0.9434	99.88	0.13	0.1497	99.69	0.18	0.5152	100.72	0.57	0.2363
30%*	100.00		1.0000	100.00		1.0000	100.00		1.0000	100.00		1.0000
31%	100.14		0.1578	100.14		0.4428	99.72		0.9222	99.59		0.4535
Acetonitrile	Confidence interval = 99.22 to 100.78%			99.83 to 100.17%			99.92 to 100.08%			98.80 to 101.20%		
4.9%	99.88	0.10	0.8205	99.83	0.09	0.0979	99.98	0.03	0.6991	99.25	0.39	0.2811
5%*	100.00		1.0000	100.00		1.0000	100.00		1.0000	100.00		1.0000
5.1%	99.79		0.6671	99.86		0.2287	99.94		0.4384	99.79		0.6951
Formic Acid	Confidence interval = 99.28 to 100.72			99.64 to 100.36%			99.68 to 100.32%			98.64 to 101.36%		
0.9%	100.08	0.11	0.5945	100.21	0.16	0.1736	100.24	0.11	0.2182	99.80	0.22	0.6903
1%*	100.00		1.0000	100.00		1.0000	100.00		1.0000	100.00		1.0000
1.1%	99.87		0.5493	99.89		0.4676	100.05		0.6255	100.24		0.7796
Temperature	Confidence interval = 98.7 to 101.24%			99.84 to 100.16%			99.71 to 100.29%			97.86 to 102.14%		
23 °C	99.92	0.06	0.3947	99.87	0.08	0.1057	100.11	0.06	0.5152	99.77	0.18	0.6206
25 °C*	100.00		1.0000	100.00		1.0000	100.00		1.0000	100.00		1.0000
27 °C	100.03		0.8861	99.84		0.1058	100.02		0.9222	100.12		0.5696
Flow Rate	Confidence interval = 97.83 to 102.17%			97.99 to 102.01%			96.58 to 102.46%			98.02 to 101.98%		
0.98 mL min^{-1}	102.03	1.96	0.9434	101.88	2.00	2.3933^{-5}	101.73	1.06	6.4554^{-5}	102.28	2.30	8.6387^{-6}
1.0 mL min^{-1}*	100.00		1.0000	100.00		1.0000	100.00		1.0000	100.00		1.0000
1.02 mL min^{-1}	98.11		8.3198^{-5}	97.88		9.5319^{-8}	97.69		1.9681^{-6}	97.68		1.5826^{-6}

Data: *Optimized conditions; **Standard concentration: Coumarin, 25.0470 μg mL^{-1}; *o*-coumaric acid, 24.8805 μg mL^{-1}; dihydrocoumarin, 24.9480 μg mL^{-1}; syringaldehyde, 24.8490 μg mL^{-1}

Table 5: Accuracy and precision by recovery test for robustness analysis (n=6).

changes in temperature, methanol and acetonitrile were performed (R_s variation = 9.32%, 5.45% and 4.96%, respectively).

However, there was no co-elution among the components when a sample of syrup (containing all interferences) was analyzed under the changed conditions. Therefore, there were no significant changes in chromatographic profile, since the separations between the peaks were maintained. Additionally, the tailing factor was calculated to evaluate the system performance and no significant change in the symmetry of the peaks was observed when the parameters were modified (RSD < 2%).

According to data presented in Table 5 for all compounds the precision was not affected (RSD < 5%) when the optimized conditions was modified. The accuracy was also maintained when variations in temperature, organic solvent and formic acid were performed. Therefore the recovery values were within the estimated confidence interval and the *p* value > 0.05 (test *t*) confirmed no significant differences between the standard concentration (obtained with optimized conditions) and the mean concentrations (obtained after small changes in the optimized conditions). The only parameter found to be critical in terms of accuracy was the flow rate (Table 5).

Sample analyses: After the method validation, twelve guaco phytomedicine samples (syrup and oral solution) as well as aqueous and hydroalcoholic extracts were analysed by the proposed method. Among these samples, aqueous extract presented 3924.3 μg mL^{-1} of coumarin, 43.3 μg mL^{-1} of *o*-coumaric acid, 2.1 μg mL^{-1} of dihydrocoumarin and 1.7 μg mL^{-1} of syringaldehyde. The hydroalcoholic tincture presented 973.6 μg mL^{-1} of coumarin, 10.2 μg mL^{-1} of *o*-coumaric acid, 1.2 μg mL^{-1} of dihydrocoumarin and 1.0 μg mL^{-1} of syringaldehyde.

For the pharmaceutical preparations, contents quite variated were achieved with individual levels ranging from 2.3 to 281.0 μg mL^{-1} for coumarin, traces to 23.7 μg mL^{-1} for *o*-coumaric acid, not detected to1.5 μg mL^{-1} for dihydrocoumarin, and not detected to 1.2 μg mL^{-1} for syringaldehyde. The results suggest a lack of standardization in the amounts of the metabolites contained in these products. Therefore the efficacy can vary considerable depending of the used sample.

Conclusions

The new method presented in this work was very effective for the simultaneous determination of coumarin, *o*-coumaric acid, dihydrocoumarin and syringaldehyde, directly in guaco extracts and phytomedicines (syrup and oral solution) without need of any sample pre-treatment. The method was found to be selective, linear, precise and accurate. The robustness study indicated that only flow rate could not be changed under the conditions of the proposed method. Sample analysis demonstrated a lack of standardization in the amounts of the main guaco metabolites among the samples. Thus the efficacy can be compromised depending of the used sample. The new method is presented as an alternative for the quality control of guaco extracts and pharmaceutical preparations.

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