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Short communication

Validation of an HPLC-DAD method for the determination of plant phenolics

Rosa M. González-González [a,1], Lina Barragán-Mendoza [a,1], Ana L. Peraza-Campos [a], Roberto Muñiz-Valencia [a], Silvia G. Ceballos-Magaña [b,*], Hortensia Parra-Delgado [a,*]

[a] Facultad de Ciencias Químicas, Universidad de Colima, Coquimatlán, Colima, México
[b] Facultad de Ciencias, Universidad de Colima, Colima, México

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ABSTRACT

A selective, sensitive and precise reversed phase HPLC-DAD method was developed and validated for the simultaneous determination of six phenolic acids in the aqueous extract and their hydrolyzed forms prepared from *Solanum elaeagnifolium* Cav., Solanaceae, *Ampelocissus acapulcensis* (Kunth) Planch., Vitaceae, or *Brosimum alicastrum* Sw., Moraceae. The new method showed good linearity ($r > 0.999$) in a relatively wide concentration range (0.5–100 mg/l). The limits of detection and quantification for the compounds were in the range of 0.097–0.467 mg/l and 0.097–0.496 mg/l, respectively. The recoveries of compounds were calculated in three different concentrations in the range of 88.07–109.17% and matrix effect was less than 5% for all phenolic acids. Finally, our developed HPLC method is simple, reliable and successfully applied to identify and quantify the phenolic acids in complex aqueous extracts from medicinal species, that can be useful for the analysis of infusions that people consume in folk medicine.

Introduction

Medicinal plants have been used in the treatment of diseases that affect the population. However, the people consume them despite the knowledge about their chemical composition is rather limited (Pires et al., 2017).

Nowadays, there is a growing interest in the analysis and identification of medicinal plants constituents, mainly phenolic compounds (Ibrahim et al., 2015). Such secondary metabolites comprise a large variety of compounds: simple flavonoids, tannins, lignin, complex flavonoids, anthocyanins, and phenolic acids (Lin et al., 2016). These latest occur in different forms such as, aglycones, esters, glycosides, and/or bound complexes (Ross et al., 2009) and they possess a broad spectrum of pharmacological actions such as antibacterial and antifungal activity, anticarcinogenic, cardioprotective, antiviral, and antiallergic (Wen et al., 2005; Abad-García et al., 2007).

As part of our studies of Mexican medicinal plants, we included the analysis of phenolic compounds of three medicinal species, *Solanum elaeagnifolium* Cav., Solanaceae, *Ampelocissus acapulcensis* (Kunth) Planch., Vitaceae, and *Brosimum alicastrum*. Sw., Moraceae.

Brosimum alicastrum (known as *mojo* or *Ramon*) is a tree considered as multipurpose species, because all its parts can be employed in different types of applications. The seeds, leaves, latex, and bark of *mojo* are medicinally used in various regions of Mexico and Guatemala. An extract of the crushed seeds is recommended as a galactogen which stimulates the milk production. Leaf infusions are employed as cough suppressants and in the treatment of kidney ailments. A tonic made from the bark is used to treat chest pains, asthma and cancer (Ortiz et al., 1995). *S. elaeagnifolium* (common name *tomatillo*) is a perennial shrub of Solanaceae family. This plant has traditionally been used for the treatment of cancer, sore throats, toothaches, and gastrointestinal disorders (Houda et al., 2014). Finally, *A. acapulcensis* (common name *uva cimarrona*) is a scandent shrub native from Mexico that has been used in traditional medicine for the treatment of disorders of the genitourinary system (Vergara-Santana et al., 2013).

The extraction of phenolic acids is carried out with aqueous-organic solvents to obtain soluble polyphenols followed by a hydrolysis treatment to obtain free polyphenols, where the acid and basic hydrolysis are the most common (Arranz and Saura-Calixto, 2010; Ozer, 2017). Several HPLC methods have been reported to quantify these compounds in extracts from plants (Sinha et al., 2007; Farzaei et al., 2014; Ozer, 2017; Pires et al., 2017). However,

* Corresponding authors.
E-mails: silvia_ceballos@ucol.mx (S.G. Ceballos-Magaña), hparra@ucol.mx (H. Parra-Delgado).
[1] Both authors contributed equally to this work.

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existing methods are used for organic extracts and there are few reports for the determination of phenolic acids in aqueous extracts (De Souza et al., 2002; Ziaková and Brandšteterová, 2003). Taking into account that several remedies are prepared as infusions, it is still important the development of methods for aqueous matrices. Therefore, in this paper, we report a new HPLC-DAD method for the determination of phenolic acids that was validated and applied to the analysis of aqueous extracts of different medicinal plants.

Materials and methods

HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from J.T. Baker-company (Guadalajara, Mexico). Ultrapure-water (Milli-Q) with 18.2 MΩ/cm resistivity was obtained from Millipak Express Filter Unit equipment (Millipore, Bedford, MA, USA). Standards of gallic (GA), vanillic (VA), *p*-hydroxybenzoic (*p*-HBA), caffeic (CA), *p*-coumaric (*p*-CoA), and *trans*-cinnamic (*trans*-CA) acids were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Fruits, leaves and bark of *B. alicastrum* were collected from Suchitlan, Colima, Mexico (19°23′55.0″N 103°45′21.9″W) in March 2015. Leaves of *S. elaeagnifolium* were obtained from Alpuyequito, Colima, Mexico (19°10′26.1″N 103°43′18.3″W) in September 2014. Root of *A. acapulcensis* were collected from Tecoman, Colima, Mexico (18°53′00.6″N 103°43′53.8″W) in May 2013. The vegetal material of three species was dried in convection oven at 37 °C for 12 h. The samples were milled using a manual mill. The flour obtained was stored in a glass hermetic recipient and stored in dark at 4 °C.

Botanical identification of three species was kindly provided by M.S. Rafael Torres-Colin of National Herbarium of Mexico (MEXU), Institute of Biology, UNAM, Mexico. Voucher herbarium specimens for *Ampelocissus acapulcensis* (Kunth) Planch., Vitaceae, (1453310), *Solanum elaeagnifolium* Cav., Solanaceae (1423235), *Brosimum alicastrum* Sw., Moraceae (1457305), were deposited at MEXU.

HPLC-DAD analyses were performed on an Alliance e2695 separation module, consisting of a quaternary pump, online vacuum degassing, autosampler, and column oven coupled to a 2996 DAD with a wavelength range of 190–800 nm from Waters (Milford, MA, USA). Data collection and analysis were performed using Empower Pro 2 Software from Waters (Milford, MA, USA). For chromatographic separation, first, two columns were used, XBridge-C18 column (4.6 mm × 150 mm, 3.5 μm) from Waters (Wexford, Ireland) and Ascentis-Express RP-Amide column (4.6 mm × 250 mm, 5 μm) from Sigma–Aldrich (St. Louis, MO, USA). For the chromatographic separations of phenolic compounds, the following conditions were employed, an Ascentis Express RP-Amide column at a 0.7 ml/min flow-rate, 25 °C, and an injection volume of 15 μl. A linear gradient profile of mobile phase A (0.1% V/V formic acid/ultrapure-water) and mobile phase B (methanol), that consisted in 15–80% B over 60 min, 15% B at 70 min. Total run time was 70 min. Identification of the phenolic compounds was achieved by comparing retention times and UV spectra of the unknowns with the standards.

Considering the ethnomedical information of each plant, different aqueous extracts were prepared. Complete information about standard solutions and preparation of the samples, and their hydrolysis (Ross et al., 2009) can be found in Supplementary data.

Validation of the proposed method was carried out according to the International Conference on Harmonization (ICH, 1996/2005) and the Commission Decision (2002/657/EC) guidelines. The method was validated through estimation of selectivity, linearity, precision, accuracy, and sensibility (Supplementary data).

The matrix effect was assessed according with the European Commission (SANTE/11945/2015) by comparing the slope of calibration curve of standards and the curve obtained after the addition of the different concentrations of the standards to the sample. The matrix effect was determined as follows (Gómez-Ramos et al., 2016):

$$\text{Matrix effect } (\%) = \left[\left(\frac{\text{slope}_{\text{standard}}}{\text{slope}_{\text{spiked}}} \right) - 1 \right] \times 100$$

Results and discussion

Some HPLC methods reported for the analysis of phenolic compounds, in medicinal plants, use organic solvents for their extraction, e.g. methanol/acetone, ethanol/water, methanol/water, ethyl acetate, butanol etc., (Ozer, 2017; Pires et al., 2017). However, most people use herbal remedies in the form of infusion or tea (Pires et al., 2017). For this reason, in the present study we proposed a HPLC method which can be used for aqueous extracts and applied for the analysis of three medicinal plants of the region, in order to identify and quantify the phenolic compounds consumed by a person when drinking the infusion. Considering that some phenolic acids are not free, it is necessary to hydrolyze them. Therefore, the extracts were sequentially hydrolyzed, first using methanol/acetic acid, then 10 M NaOH and finally concentrated HCl (Ross et al., 2009).

For the optimization of the method several conditions, solvents, times and column types were used to obtain the optimum separation of each standard in the extracts. Two mobile phases were tested, MeOH:H_2O/formic acid (0.1%) and ACN:H_2O/phosphoric acid (0.1%). Both mobile phases allowed the correct separation of the standards, however, MeOH:H_2O/formic acid (0.1%) was the phase with better resolution and separation of phenolic compounds within the aqueous extracts. Besides, various flow rates (0.5, 0.7, 1 and 1.2 ml/min) and column types (XBridge-C18 column and Ascentis-RP amide column) were employed. XBridge-C18 presented an overlap of phenolic acids. Using the Ascentis-RP amide, all the phenolic acids were baseline separated with a total analysis time of 70 min. Chromatograms were acquired at different wavelengths according to absorption maxima of analyzed compounds. These were 270, 292, 260, 324, 310, and 277 nm for GA, VA, *p*-HBA, CA, *p*-CoA, and *trans*-CA, respectively.

The established HPLC method was validated in terms of selectivity, linearity, precision, accuracy, and sensibility according to ICH and Commission Decision guidelines (ICH, 1996/2005; 2002/657/EC). The validation parameters are shown in Table 1.

Method selectivity was evaluated by overlapping the chromatogram of the standard solution with that of the samples. Also, the comparison of the UV spectra was carried out (Fig. 1).

Linearity was evaluated by using standard solutions dissolved in 15:85 mobile phase B/A at concentrations in the range of 5–75 mg/l for GA and *p*-HBA, 1–75 mg/l for VA, 0.5–100 mg/l for CA, 1–100 mg/l for *p*-CoA, and 2.5–50 mg/l for *trans*-CA. Each concentration was analyzed in triplicate. The calibration curves were constructed from peak areas of the reference compounds versus their concentrations (Fig. 1A, Supplementary data). The correlation coefficients of all the calibration curves were greater than 0.999. The precision of the method was determined as repeatability and reproducibility in terms of percent relative standard deviation (%RSD). The %RSD values for evaluated concentrations (10, 25 and 50 mg/l) were lower than 2.18%. Recovery was determined employing the extracts in which the peaks were clear and without interferences. Therefore, for GA the process was made in the leaves total extract of *B. alicastrum;* meanwhile for VA, *p*-HBA, CA, *p*-CoA, and *trans*-CA acids the procedure was carried out in the "basic hydrolysis" of *B. alicastrum*. The results obtained show percentages of recovery between 88.07-109.17. These

Table 1
Validation parameters for the HPLC–DAD method used for the determination of phenolic acids in different extracts of medicinal plants.

Analyte	Precision (RSD[g] %)		Accuracy											Matrix effect (%)
	t_R[a] (min)	Linear range (mg/l)	r[b]	m[c]	b[d]	LOD[e] (mg/l)	LOQ[f] (mg/l)	Repeatability			Reproducibility			Recovery (%)			
								C_1[h]	C_2[i]	C_3[j]	C_1[h]	C_2[i]	C_3[j]	C_1[h]	C_2[i]	C_3[j]	
GA	11.86	5–75	0.9998	31616	−24109	0.4665	0.4958	1.17	0.65	2.18	1.13	0.31	1.29	106.40	91.18	95.22	−3.18
VA	27.19	1–75	0.9997	60045	−8584.2	0.1181	0.1244	0.47	0.46	1.14	0.68	0.30	0.09	103.58	97.18	97.79	1.26
p-HBA	28.89	5-75	0.9996	60696	−21411	0.4161	0.4767	0.40	0.29	0.17	0.53	0.21	0.08	99.96	88.07	97.94	−4.75
CA	30.00	0.5–100	0.9996	243407	−17962	0.0965	0.0967	0.57	1.05	1.49	0.41	0.21	0.27	108.31	103.03	102.07	0.55
p-CoA	37.97	1–100	0.9995	93693	−27383	0.2758	0.2904	0.39	0.81	0.15	0.55	0.82	0.66	109.17	101.23	102.31	2.13
trans-CiA	45.49	2.5–50	0.9995	159044	−203414	0.1721	0.1816	0.21	0.29	0.05	0.41	0.11	0.04	96.88	102.49	101.12	1.96

GA, gallic acid; VA, vanillic acid; *p*-HBA, *p*-hydroxybenzoic acid; CA, caffeic acid; *p*-CoA, *p*-coumaric acid; *trans*-CA, *trans*-cinnamic acid.
[a] Retention time (min).
[b] Correlation coefficient.
[c] Slope.
[d] Intercept.
[e] Limit of detection.
[f] Limit of quantification.
[g] Relative standard deviation.
[h] Low concentration level of 10 mg/l.
[i] Middle concentration level of 25 mg/l.
[j] High concentration level of 50 mg/l.

Fig. 1. HPLC chromatograms obtained for the standard phenolic compounds employed in the optimization of chromatographic method at 270 nm (A). Total aqueous extract (B) and basic hydrolysis (C) of leaves *Brosimum alicastrum* Swartz. GA: gallic acid; VA: vanillic acid; *p*-HBA: *p*-hydroxybenzoic acid; CA: caffeic acid; *p*-CoA: *p*-coumaric acid; *trans*-CA: *trans*-cinnamic acid.

precision and recovery values are considered acceptable according to Commission Decision (2002/657/EC).

LOD values were calculated between 0.097 and 0.467 mg/l and the LOQ values were found in the range of 0.097–0.496 mg/l, indicating high sensibility of the method.

Matrix effect (ME) is considered the response of an analyte, either positive or negative, caused by coeluting compounds, relative to an injection of a pure standard, and its presence is related to the sample nature, affecting reproducibility, linearity, and accuracy of the methods (Cappiello et al., 2010). Because of extracts are usually constituted by complex mixture of compounds, in this work we considered important to include a ME study. It was explored by comparing the external calibration slope with the matrix match calibration slope. The results obtained showed signal suppression for GA and *p*-HBA, while there was a signal enhancement for VA, CA, *p*-CoA and *trans*-CA. In all cases, it was less than

Table 2
Content of phenolic acids in aqueous extracts of *Brosimum alicastrum* (leaves, bark and seeds), *Solanum elaeagnifolium* (leaves) and *Ampelocissus acapulcensis* (root) and their hydrolysis.

	GA (μg/mg of lyophilizate extract)	VA (μg/mg of lyophilizate extract)	*p*-HBA (μg/mg of lyophilizate extract)	CA (μg/mg of lyophilizate extract)	*p*-CoA (μg/mg of lyophilizate extract)	*trans*-CiA (μg/mg of lyophilizate extract)
B. alicastrum leaves						
Total extract	2.34 ± 0.007	n/d	n/d	n/d	n/d	n/d
MeOH/AcA hydrolysis	0.91 ± 0.081	n/d	n/d	0.15 ± 0.038	n/d	n/d
Basic hydrolysis	n/d	1.44 ± 0.010	n/d	0.71 ± 0.0048	n/d	n/d
B. alicastrum bark						
Total extract	n/d	n/d	n/d	n/d	n/d	n/d
MeOH/AcA hydrolysis	n/d	n/d	n/d	n/d	n/d	n/d
Basic hydrolysis	n/d	19.44 ± 0.253	11.55 ± 0.054	n/d	n/d	n/d
Acid hydrolysis	n/d	0.58 ± 0.022	0.30 ± 0.002	0.06 ± 0.001	n/d	n/d
B. alicastrum seed						
Total extract	n/d	0.65 ± 0.005	n/d	n/d	n/d	n/d
MeOH/AcA hydrolysis	n/d	n/d	n/d	n/d	n/d	n/d
Basic hydrolysis	n/d	0.10 ± 0.014	n/d	0.0058 ± 0.002	0.013 ± 0.0004	n/d
Acid hydrolysis	n/d	0.09 ± 0.012	n/d	n/d	n/d	n/d
S. elaeagnifolium leaves						
Total extract	n/d	n/d	n/d	n/d	n/d	n/d
MeOH/AcA hydrolysis	n/d	n/d	n/d	0.04 ± 0.033	0.01 ± 0.006	n/d
Basic hydrolysis	n/d	0.35 ± 0.015	0.77 ± 0.015	0.11 ± 0.003	0.89 ± 0.014	n/d
Acid hydrolysis	n/d	0.22 ± 0.025	0.32 ± 0.009	0.10 ± 0.0003	n/d	n/d
A. acapulcensis root						
Total extract	6.01 ± 0.023	n/d	n/d	n/d	n/d	n/d
MeOH/AcA hydrolysis	1.47 ± 0.180	n/d	n/d	n/d	n/d	n/d
Basic hydrolysis	5.34 ± 0.052	n/d	1.57 ± 0.008	n/d	3.35 ± 0.057	n/d
Acid hydrolysis	n/d	n/d	0.51 ± 0.006	n/d	1.45 ± 0.013	n/d

GA, gallic acid; VA, vanillic acid; *p*-HBA, *p*-hydroxybenzoic acid; CA, caffeic acid; *p*-CoA, *p*-coumaric acid, *trans*-CA: *trans*-cinnamic acid, n/d, not detected.

5% which can be accepted according with European Commission (SANTE/11945/2015).

In order to demonstrate the applicability of the developed method, three different medicinal plants were analyzed. The content of phenolic acids in aqueous total extracts and their hydrolyzed forms is shown in Table 2. Phenolic compounds that were found free in the total aqueous extracts were the GA (2.34 μg/mg LE) in the leaves of *B. alicastrum* (Fig. 1) and in the root of *A. acapulcensis* (6.01 μg/mg LE) (Fig. 4A–E1, Supplementary data), while vanillic acid (0.65 μg/mg LE) was found in the seeds of *B. alicastrum* (Figure 3A–C1, Supplementary data). Furthermore, the new method allowed the determination and quantification of the phenolics in the hydrolyzed. Our results indicated that most of the phenolic acids in the extracts are joined to other compounds forming insoluble bound complexes (Ross et al., 2009).

Conclusion

The HPLC method for the analysis of six phenolic acids in total aqueous extracts of *B. alicastrum*, *S. elaeagnifolium* and *A. acapulcensis* and their hydrolyzed forms was developed and validated. The method showed good linearity, precision, sensibility, selectivity, and recovery. In addition, the matrix effect was <5% for all phenolic acids. Thus, the developed method is simple, reliable and successfully applied to identify and quantify phenolic acids in complex aqueous extracts from diverse parts of a plant and different medicinal species.

Authors' contributions

LBM and RMGG contribution included HPLC analysis, analyzing the results and preparing the paper. SGCM, RMV, ALPC and HPD contribution included analyzing results and preparing the paper. All authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical disclosures

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.06.002.

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