Journal of Chromatographic Science, 2018, Vol. 56, No. 4, 300–306
doi: 10.1093/chromsci/bmx111
Advance Access Publication Date: 29 December 2017
Article

Article

Development and Validation of a Reversed Phase HPLC Method for Determination of Anacardic Acids in Cashew (*Anacardium occidentale*) Nut Shell Liquid

Francisco Oiram Filho[1], Daniel Barbosa Alcântra[2], Tigressa Helena Soares Rodrigues[3], Lorena Mara Alexandre e Silva[3], Ebenezer de Oliveira Silva[3], Guilherme Julião Zocolo[3], and Edy Sousa de Brito[3,*]

[1]Department of Chemical Engineering, UFC, Federal University of Ceará, Campus do Pici, Bloco 709, 60455-760, Fortaleza, CE, Brazil, [2]Department of Chemistry, UFC, Federal University of Ceará, Campus do Pici, 60455-760, Fortaleza, CE, Brazil, and [3]Embrapa Agroindústria Tropical, R Dra Sara Mesquita, 2270, Pici, 60511-110, Fortaleza, CE, Brazil

[*]Author to whom correspondence should be addressed. Email: edy.brito@embrapa.br

Received 11 April 2017; Revised 27 October 2017; Editorial Decision 5 December 2017

Abstract

Cashew nut shell liquid (CNSL) contains phenolic lipids with aliphatic chains that are of commercial interest. In this work, a chromatographic method was developed to monitor and quantify anacardic acids (AnAc) in CNSL. Samples containing AnAc were analyzed on a high-performance liquid chromatograph coupled to a diode array detector, equipped with a reversed phase C_{18} (150 × 4.6 mm × 5 μm) column using acetonitrile and water as the mobile phase both acidified with acetic acid to pH 3.0 in an isocratic mode (80:20:1). The chromatographic method showed adequate selectivity, as it could clearly separate the different AnAc. To validate this method, AnAc triene was used as an external standard at seven different concentrations varying from 50 to 1,000 μg mL^{-1}. The Student's *t*-test and *F*-test were applied to ensure high confidence for the obtained data from the analytical calibration curve. The results were satisfactory with respect to intra-day (relative standard deviation (RSD) = 0.60%) and inter-day (RSD = 0.67%) precision, linearity ($y = 2{,}670.8x - 26{,}949$, $r^2 > 0.9998$), system suitability for retention time (RSD = 1.02%), area under the curve (RSD = 0.24%), selectivity and limits of detection (19.8 μg mg^{-1}) and quantification (60.2 μg mg^{-1}). The developed chromatographic method was applied for the analysis of different CNSL samples, and it was deemed suitable for the quantification of AnAc.

Introduction

The cashew tree (*Anacardium occidentale*) has two main products, the cashew apple (a pseudo-fruit that can be consumed as a fruit or juice) and the cashew nut (fruit). The cashew nut is a product of commercial importance worldwide. The global production of cashew nuts in 2014 was ~3.8 million ton (1). Processing of the fruit results in nuts (30%) and a large amount of shells (70%), considered to be a byproduct in the industry. Cashew nut shells have an internal part called the pericarp, with a honeycomb-like structure, containing a dark brown viscous liquid known as cashew nut shell liquid (CNSL) (2). CNSL is of commercial value because of its alkyl phenol content, which is composed of anacardic acids (AnAc), cardols and cardanols (3). AnAc have been reported to show activity against apoptosis (4), Alzheimer's disease (5), Chagas disease (6) and bacterial infection (7)

as well as antioxidant activity (8). The quantification of AnAc in CNSL is important because of the growing number of applications of this class of compounds. Methods have been developed to monitor AnAc content (9–11), but they are either time consuming or have not been validated. Therefore, the aim of this work was to develop a chromatographic method capable of quantifying these compounds using high-performance liquid chromatography (HPLC).

Experimental

Reagents and materials

CNSL was obtained by pressing the shells obtained from a processing unit of Embrapa Agroindústria Tropical (Pacajus, CE). The reagents used were acetonitrile (purity ≥ 99.9%) and methanol (purity ≥ 99.9%), both obtained from Tedia (Fairfield, Ohio, EUA); glacial acetic acid P.A. (purity ≥ 99.7%) and methanol P.A. (purity ≥ 99.8%), both obtained from Sigma-Aldrich (Saint Louis, MO, EUA); and purified water from a Mili-Q system (Millipore, São Paulo, Brazil). The external standard (AnAc, 15:3, purity ≥ 98.5%) was kindly provided by Dr Maria Teresa Sales Trevisan (Universidade Federal do Ceará).

HPLC system

The chromatographic system consisted of a Shimadzu LC-20AB Prominence Chromatograph coupled to a Shimadzu SPD-M20A Prominence Diode Array Detector and a Shimadzu SIL-20AC Prominence Autosampler (Kyoto, Japan). Instrumental control and data processing were performed using the Shimadzu LC Solution software.

Methods

Development of the chromatographic method using HPLC-DAD

Some parameters were adjusted to identify the optimal chromatographic conditions. Different chromatographic conditions were tested by changing the following parameters: C_8 (100 × 4.6 mm × 5 µm, Waters) and C_{18} (150 × 4.6 mm × 5 µm, Shimadzu) columns, concentration of mobile phase (60, 70 and 80% of acetonitrile or methanol in water), presence and absence of acetic acid (1% *v/v*), and different flow rates (1.0, 1.2 and 1.5 mL min^{-1}).

Optimization of the method was performed using a reverse-phase C_{18} chromatographic column Shim-pack CLC–ODS (M) (150 × 4.6 mm × 5 µm, Shimadzu). The mobile phase used was water (Solvent A), acetonitrile (Solvent B), and acetic acid in a ratio of 20:80:1 in isocratic mode. The analysis time was 30 min, with a flow of 1.5 mL min^{-1}, at 25°C with an injection volume of 20 µL. The chromatograms were monitored at a wavelength of 280 nm and the UV spectra were recorded from 200 to 400 nm. All chromatographic parameters mentioned were selected based on previous optimization experiments.

Structural confirmation of AnAc by UPLC–QTOF–MS^E

In order to confirm the structure of the AnAc, the samples were analyzed on an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a quadrupole/time-of-flight (QToF) mass spectrometer (Waters, Milford, MA, USA). The compounds were separated on an Acquity BEH C18 (150 × 2.1 mm^2, 1.7 µm; Waters, Milford, MA, USA) column operated at 40°C. The eluent system employed was a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.4 mL min^{-1}. The gradient varied linearly from 5 to 95% B (*v/v*) over 20 min. The sample injection volume was 5 µL. Mass spectra were obtained in the negative-ion mode over a mass range between 50 and 1180 Da. The spectrometer was operated with MS^E centroid programming using a cone voltage of 40 V. The drying gas pressure was 35 psi at 370°C, while the nebulizer gas pressure was 40 psi. A capillary voltage of 3500 V and a 600 V spray shield voltage were used.

Identification of AnAc was carried out using molecular formulas and *m/z* values obtained from high-resolution mass spectra, using the MassLynx software (Waters Corporation). The data were compared with those presented in previous reports (10, 12).

Method validation

The validation protocol was established according to the method proposed by the International Conference Harmonization or ICH (13), based on selectivity, linearity, precision (intra-day and inter-day), repeatability and limits of detection (LOD) and quantification (LOQ). An analytical curve was obtained with seven different concentrations (50, 100, 200, 400, 600, 800 and 1,000 µg mL^{-1} in methanol) of AnAc triene (15:3), typically the major AnAc constituent in CNSL. Additionally, five samples of CNSL, from different cashew clones produced in the Embrapa Agroindústria Tropical experimental station (Pacajus, CE, Brazil), were weighed and dissolved in methanol to a concentration of 1 mg mL^{-1} for the quantification of the AnAc.

Selectivity

Selectivity refers to the extent to which a method can discriminate between particular analytes in mixtures or matrices without interference from other components (14). Analyzing the 3-point purity of each peak and comparing the peak UV–Vis spectrum obtained from the standard and the peaks for the analyzed compounds was used to assess the selectivity of the method.

Linearity

To determine the linearity, AnAc (15:3) was dissolved in methanol at different concentrations (50, 100, 200, 400, 600, 800 and 1,000 µg mL^{-1}) and tests were performed in triplicate. Th Student's *t*-test and *F*-test were applied at a 95% confidence level to provide a higher confidence for data obtained from the calibration curve and linear regression. Calculations were performed using the following equations:

$$S^2a = \frac{S^2y \cdot n}{D} \quad (1)$$

$$S^2b = \frac{S^2y \cdot \sum(x^2i)}{D} \quad (2)$$

$$S^2y = \frac{\sum(d^2i)}{n-2} \quad (3)$$

where S_a is the standard deviation of the angular coefficient (a); S_b is the standard deviation of the linear coefficient (b); S_y is the standard deviation of the y-axis; x_i is the individual value of x; n is the total number of points on the curve; d_i is the vertical deviation of each point; and D is the determinant, given by $\begin{vmatrix} \sum(x_i^2) & \sum x_i \\ \sum x_i & n \end{vmatrix}$.

Starting from the calculations of different standard deviations, the values of $t_{calc(a)}$, $t_{calc(b)}$ and F_{calc} were calculated according to the following equations:

$$t_{calc(a)} = \frac{|1 - a|}{S_a} \quad (4)$$

$$t_{calc(b)} = \frac{|b|}{S_b} \quad (5)$$

$$F_{calc} = \frac{S_y}{\frac{1}{\frac{\sum d_i}{3}}} \quad (6)$$

If $t_{calc} > t_{tab}$, the parameter is significant at a 95% confidence level and should be retained. On the other hand, if $t_{calc} < t_{tab}$, the parameter is not significant and can be removed from the equation. The same procedure was applied for the *F*-test, where the values of $F_{cal} > F_{tab}$ were significant at a 95% confidence level for the linear regression and values of $F_{cal} < F_{tab}$ had no significance; therefore, no linear relationship was obtained between the *x* and *y* axes, independent of the values for the correlation coefficient, r^2.

Precision

To determine the precision of the method, the reproducibility of seven different concentrations (50, 100, 200, 400, 600, 800 and 1,000 μg mL^{-1}) was evaluated. The reproducibility of triplicates of each point on the standard curve was analyzed on the same day (intra-day) and on consecutive days (inter-day). The precision was obtained as a function of the standard deviation and relative standard deviation, according to the equations (7) and (8):

$$S = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n - 1}} \quad (7)$$

where *S* is the standard deviation, *n* is the number of measurements and x_i is the difference between each measured value and $\bar{x}$ is the average of all measurements.

$$CV = \frac{S}{\bar{x}} \cdot 100 \quad (8)$$

System suitability test

To assess the repeatability of the method, an intermediate concentration point of the calibration curve was injected 10 times consecutively. The retention time and areas obtained were used to calculate the standard deviation and relative standard deviation.

LOD and LOQ

The limits of detection and quantification were estimated according to the minimum concentration of each parameter, following equations (9) and (10). Therefore, for LOD, a minimum concentration was established to differentiate an analyte from noise. For LOQ, the minimum concentration was the lowest concentration at which an analyte could be quantified accurately.

$$LOD = 3{,}3 \cdot \frac{S_b}{a} \quad (9)$$

$$LOQ = 10 \cdot \frac{S_b}{a} \quad (10)$$

where S_b is the standard deviation at the *Y*-intercept and *a* is the angular coefficient of the calibration curve (13, 15).

Results

The chromatographic separation conditions employed were based on the separation conditions previously established by Paramashivappa *et al.* (9) to evaluate the alkyl phenols presents in CNSL. Exploratory experiments were performed to achieve the best separation results. The best results were achieved with a C18 column, a mobile phase with 80% of acetonitrile with acetic, and a flow rate of 1.5 mL min^{-1}. The peaks were identified using their retention times and UV spectra. Figure 1a shows the chromatographic profiles of CSNL, where peaks I–V were assigned to cardol, cardanol and the AnAc C15:3, C15:2 and C15:1, respectively. Figure 1b shows the chromatographic profile of the external standard of AnAc triene (15:3). Selectivity was evaluated by analyzing the UV spectrum of the external standards used. The purities, obtained as a mean of three values, of the AnAc were 99.98, 99.98 and 99.97% for the (15:3), (15:2) and (15:1) species, respectively. The identity of the peaks was confirmed by their characteristic UV spectra (Figure 2). Throughout the chromatogram, no interference was detected.

The analytical calibration curve was prepared with seven different concentrations (50, 100, 200, 400, 600, 800 and 1,000 μg mL^{-1}) of AnAc (15:3). Table I shows good linearity within the analyzed range, which was confirmed by the correlation coefficient ($y = 2670.8x - 26949$; $r^2 = 0.9998$). The correlation coefficient was in agreement with the accepted values ($r^2 \geq 0.99$) (11). The significance of the Student's *t*-test for the calibration parameters, as well as the significance of the *F*-test for the linear regression, assured a higher confidence for the data obtained from the analytical curve (Table I). Both Student's *t*-test and *F*-test were analyzed at the 95% confidence level.

The precision of the method was evaluated by the relative standard deviation (RSD) and correlation coefficient (r^2) obtained for the values of the standard retention time and the data obtained from the inter-day and intra-day tests. The values of the correlation coefficient and relative standard deviation of the intra-day and inter-day injections were within generally acceptable values (Table I). All relative standard deviation values were in agreement with the values established by the ICH (13). The values of RSD acquired from 10 consecutive injections of a known concentration are shown in Table I. The retention times and peak area values obtained showed RSDs of 1.02 and 0.24%, respectively. There is no generally accepted rule for allowable limits of deviation, but deviation values close to 1% for retention time and peak area are usually acceptable (16, 17). The LOD and LOQ of the AnAc at 280 nm were 19.8 and 60.2 μg mg^{-1}, respectively.

Table II shows the identification of three different AnAc in the CNSL samples. AnAc triene, diene and monoene were confirmed according to their masses and respective ionization fragments identified by mass spectrometry. The molecular formulas were obtained by analyzing their first order mass spectra and all of the respective errors were <10 ppm. The chemical structures were confirmed

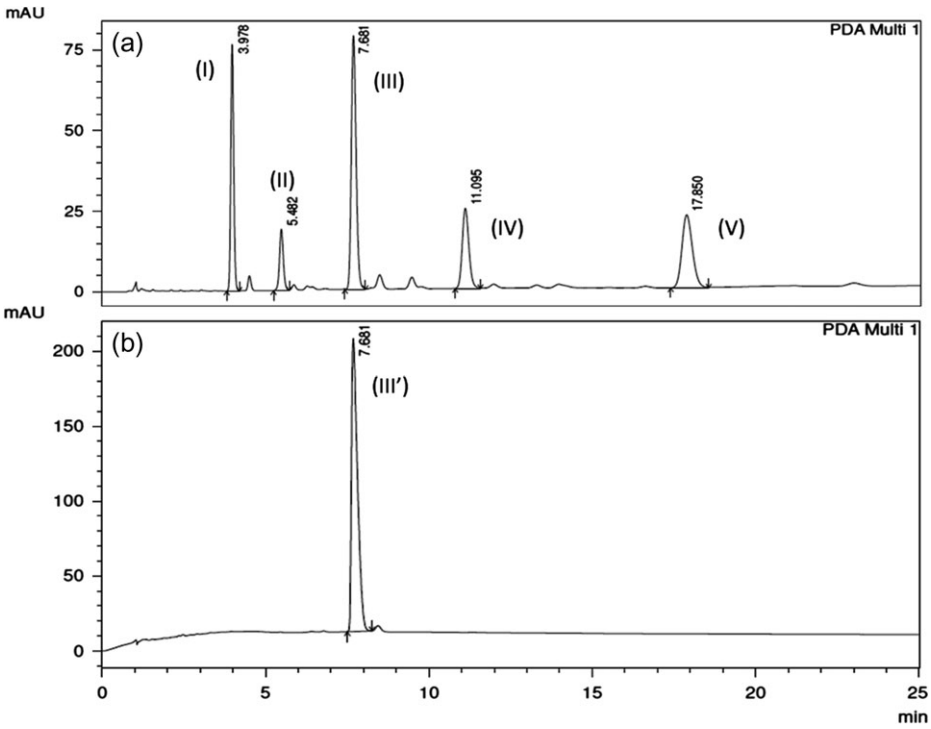

Figure 1. (a) Chromatographic profile of CNSL (1 mg mL^{-1}) monitored at 280 nm for cardol (I); cardanol (II); anacardic acid triene (III), diene (IV) and monoene (V). (b) Chromatographic profile for the external standard anacardic acid triene (1 mg mL^{-1}) monitored at 280 nm (III′).

through gas phase fragmentation of second order mass spectra, where the characteristics fragment ions were observed for each AnAc triene, diene and monoene. The AnAc 15:3 was identified by a $[M–H]^-$ of 341.2094 Da and an ion fragment with a *m/z* of 297.2222 Da; the 15:2 species was identified by a $[M–H]^-$ of 343.2245 Da and an ion fragment *m/z* 299.2355 Da; the 15:1 species was identified by a $[M–H]^-$ of 345.2409 Da, along with a fragment *m/z* 301.2534 Da. The fragmentation of $[M–H-44]^-$ for all the three identified compounds, owing to the loss of CO_2 due to the cleavage of the carboxylic group and all the fragments agreed well with previous results (10, 18, 19).

Five samples of CNSL from different batches were quantified indirectly using the external standard to quantify the AnAc present in the samples (Table III). The external standard shows the same maximum UV absorption as the AnAc present in the samples analyzed. The sample CNSL 701 showed concentrations of 351.2, 133.7 and 221.1 μg mg^{-1} for AnAc triene, diene and monoene, respectively. CNSL 704 exhibited concentrations of 326.5, 273.2 and 255.0 μg mg^{-1} for AnAc triene, diene and monoene, respectively. CNSL 709 showed corresponding concentrations of 299.9, 231.6 and 203.9 μg mg^{-1}, respectively. CNSL 710 had 308.0, 141.4 and 198.8 μg mg^{-1} of AnAc triene, diene and monoene, respectively. The corresponding concentrations found in the sample CNSL 715 were 311.2, 221.6 and 187.2 μg mg^{-1}.

Discussion

During the optimization studies, the C_{18} column showed best chromatographic resolution compared to the C_8 column, achieving separate and distinct peaks for the different AnAc. The AnAc are inherently very non-polar, so these compounds were better separated on the C_{18} column because it contains a more non-polar adsorbent compared to the C_8 column (20). Acetonitrile was chosen as an organic solvent due its higher elution strength compared to methanol (21). A proportion of 80% acetonitrile together with a flow rate of 1.5 mL min^{-1} were chosen as the optimal conditions based on the shortest run time and minimal concentration which achieved perfect separation of each compound in CNSL. The presence of acetic acid in the mobile phase (pH 3.0) was necessary to suppress the residual silanol present in the column (22), thus ensuring good resolution and retention time of the chromatographic peaks (23, 24).

In comparison with the validation of other chromatographic methods (25–28), the developed method was effective in monitoring, separating and quantifying the three types of AnAc present in CNSL. The elution sequence of the AnAc was triene (15:3) followed by diene (15:2) and monoene (15:1). This was due to the stationary phase (reverse-phase), where the most polar AnAc triene (15:3) was expected to elute first. However, the opposite occurs in a normal phase chromatographic column, where the first AnAc eluted was the saturated species (15:0) (29).

The quantification of the AnAc was performed in an indirect way due to the structural similarity between the quantified compounds and the analytical standard used. The UV spectra acquired for each of the analyzed compounds showed a profile and maximum absorption equivalent to that of the external standard used (Figure 2), and also matched those previously reported in the literature (29, 30). The similarity of the absorptions bands for the AnAc occurs due to their structural similarity, the only difference being the extent of unsaturation in the side chain of the molecule (Figure 3).

The major AnAc product was the saturated 15:0 species at a concentration of 416 μg mg^{-1}. The concentrations and AnAc profile

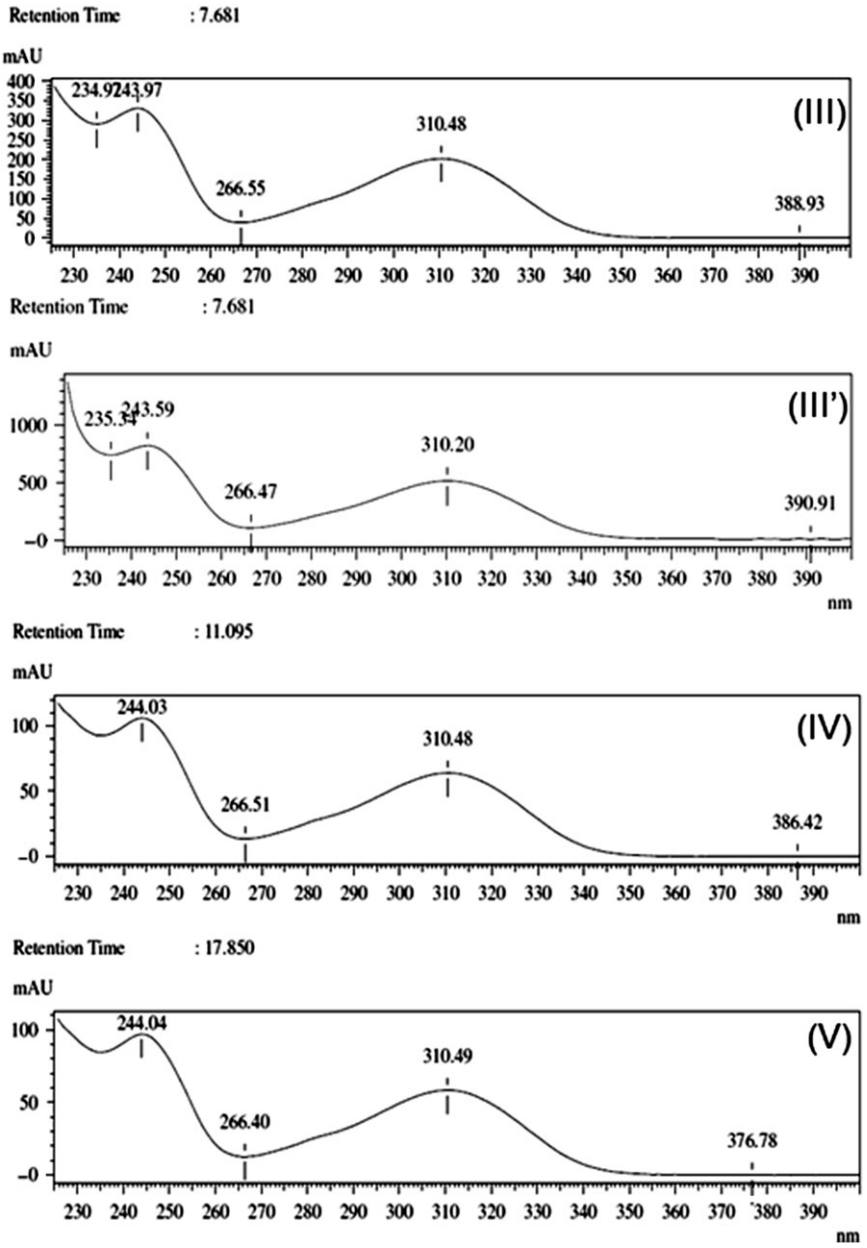

Figure 2. UV spectrum for anacardic acid (III) triene, (IV) diene and (V) monoene and the triene external standard (III′).

Table I. Anacardic Acid (15:3) Analytical Parameters and Statistical Results of Linear Regression, Results of Intra-day and Inter-day Precision and Repeatability

Equation	$y = 2{,}670.8x - 26{,}949$			$R^2 = 0.998$	$F_{cal} = 72974$ $F_{tab} = 2.7$
	A	S_a	t_{cal}	t_{tab}	t-test
Angular coefficient	2670.8	9.88	270.03	2.179	Significant
	b	S_b	t_{cal}	t_{tab}	t-test
Linear coefficient	26949	5558.48	4.85	2.179	Significant
		R^2	RSD (%)		
Intra-day ($N = 6$)		0.9998	0.60		
Inter-day ($N = 9$)		0.9979	0.67		
		Retention time (RSD %)		Area (RSD %)	
Suitability system test		1.02		0.24	

Table II. Identification of the Different Anacardic Acids (Triene, Diene and Monoene) in Cashew Nut Shell Liquid by UPLC–QTOF-MS(/MS) Analysis

Compound	$[M–H]^-$ Experimental	$[M–H]^-$ Theorical	Error (ppm)	Fragments (*m/z*)	Molecular formula
AnAc (15:3)	341.2094	341.2117	2.3	297.2222	$C_{22}H_{30}O_3$
AnAc (15:2)	343.2245	343.2273	8.2	299.2355	$C_{22}H_{32}O_3$
AnAc (15:1)	345.2409	345.2430	6.1	301.2534	$C_{22}H_{34}O_3$

Table III. Concentration of Anacardic Acids in Cashew Nut Shell Liquid (CNSL)

Samples	Anacardic acids ± SD ($\mu g\ mg^{-1}$)		
	(15:3)	(15:2)	(15:1)
CNSL 701	351.2 ± [0.8]	133.7 ± [0.7]	221.1 ± [2.7]
CNSL 704	326.5 ± [0.2]	273.2 ± [1.7]	255.0 ± [1.7]
CNSL 709	299.9 ± [2.7]	231.6 ± [2.3]	203.9 ± [2.6]
CNSL 710	308.0 ± [1.2]	1414 ± [2.2]	198.8 ± [2.9]
CNSL 715	311.2 ± [3.3]	221.6 ± [2.2]	187.2 ± [3.9]

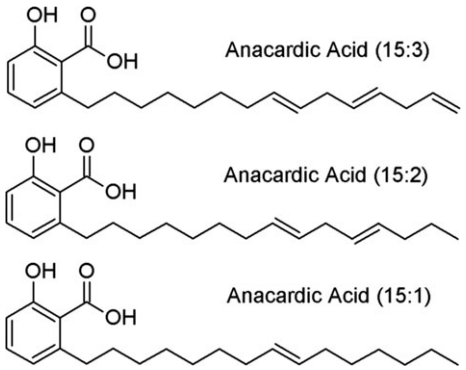

Figure 3. Molecular structure of anacardic acid triene, diene and monoene.

variations for the different CNSL samples can be attributed to genetic diversity, since all samples came from the same experimental field with similar ages and cultivation practices. The concentration of AnAc 15:3 was higher than that of the 15:2 and 15:1 species in all samples analyzed, and matched the concentration range and profile previously observed by Trevisan *et al.* (10). However, another study using countercurrent chromatography obtained the same concentration and profile only for the 15:3 and 15:2 AnAc species at 318 and 221 $\mu g\ mg^{-1}$, respectively, as reported by Jerz *et al.* (18).

Conclusion

The chromatographic method developed in this study is able to quantify alkyl phenols present in CNSL in a qualitative and quantitative manner using HPLC techniques. The method validation showed the feasibility of using an external standard to monitor and quantify the AnAc.

The chromatographic method developed herein was superior to other methods described in the literature, as it can quickly and accurately separate and quantify AnAc in the samples of CNSL, and hence, it can become the standard method for AnAc analysis in CNSL.

Funding

This work was supported by Empresa Brasileira de Pesquisa Agropecuária, Embrapa [Grant number 03.14.03.009.00.00] and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico, FUNCAP [BMD-0008-00640.01.12/15] to F.O.F.

References

1. FAO; Statistic Division. In: Food and Agriculture Organization of the United Nations (2014).
2. Gandhi, T., Patel, M., Dholakiya, B.; Studies on effect of various solvents on extraction of cashew nut shell liquid (CNSL) and isolation of major phenolic constituents from extracted CNSL; *Journal of Natural Products Plant Resources*, (2012); 2(1): 135.
3. Yuliana, M., Tran-Thi, N.Y., Ju, Y.; Effect of extraction methods on characteristic and composition of Indonesian cashew nut shell liquid; *Industrial Crops and Products*, (2012); 35(1): 230–236.
4. Huang, H., Hua, X., Liu, N., Li, X., Liu, S., Chen, X., *et al.*; Anacardic acid induces cell apoptosis associated with induction of ATF4-dependent endoplasmic reticulum stress; *Toxicology Letters*, (2014); 228(3): 170–178.
5. Lemes, L.F.N., Ramos, G.A., de Oliveira, A.S., da Silva, F.M.R., Couto, G.C., Boni, M.S., *et al.*; Cardanol-derived AChE inhibitors: towards the development of dual binding derivatives for Alzheimer's disease; *European Journal of Medicinal Chemistry*, (2016); 108: 687–700.
6. de Oliveira, V.M., Mendes, L.M., Almeida, D.J., Hoelz, L.V.B., Torres, P. H.M., Pascutti, P.G.; New treatments for Chagas disease and the relationship between Chagasic patients and cancers; *Cancer Research Journal*, (2014); 2(6-1): 11–29.
7. Hollands, A., Corriden, R., Gysler, G., Dahesh, S., Olson, J., Ali, S.R., *et al.*; Natural product anacardic acid from cashew nut shells stimulates neutrophil extracellular trap production and bactericidal activity; *Journal of Biological Chemistry*, (2016); 291(27): 13964–13973.
8. Masuoka, N., Shimizu, K., Kubo, I.; *Antioxidant Activity of Anacardic Acids. Natural Antioxidants and Biocides From Wild Medicinal Plants*. Wallingford, Oxfordshire OX10 8DE, UK, Cabi, Nosworthy Way, (2013); pp. 137–147.
9. Paramashivappa, R., Kumar, P.P., Vithayathil, P.J., Rao, A.S.; Novel method for isolation of major phenolic constituents from cashew (*Anacardium occidentale* L.) nut shell liquid; *Journal of Agricultural and Food Chemistry*, (2001); 49(5): 2548–2551.
10. Trevisan, M.T.S., Pfundstein, B., Haubner, R., Würtele, G., Spiegelhalder, B., Bartsch, H., *et al.*; Characterization of alkyl phenols in cashew (*Anacardium occidentale*) products and assay of their antioxidant capacity; *Food and Chemical Toxicology*, (2006); 44(2): 188–197.
11. Agil, R., Oomah, D.B., Mazza, G., Hosseinian, F.S.; Optimization of alkylresorcinols extraction from triticale bran using response surface methodology; *Food and Bioprocess Technology*, (2012); 5(7): 2655–2664.
12. Bouslimani, A., Sanchez, L.M., Garg, N., Dorrestein, P.C.; Mass spectrometry of natural products: current, emerging and future technologies; *Natural Product Reports*, (2014); 31(6): 718–729.
13. ICH; ICH Harmonized Tripartite: validation of analytical procedures: text and methodology. Q2 (R1) (2005); 1.
14. Vessman, J., Stefan, R.I., Van Staden, J.F., Danzer, K., Lindner, W., Burns, D.T., *et al.*; Selectivity in analytical chemistry (IUPAC

Recommendations 2001); *Pure and Applied Chemistry*, (2001); 73(8): 1381–1386.
15. Ribani, M., Bottoli, C.B.G., Collins, C.H., Jardim, I.C.S.F., Melo, L.F.C.; Validation for chromatographic and electrophoretic methods; *Química Nova*, (2004); 27: 771–780.
16. Fabre, H.; Validation des méthodes d'électrophorèse capillaire appliquées à l'analyse des composés pharmaceutiques; *Analusis*, (1999); 27(2): 155–155.
17. Lanças, F.M.; Validação de métodos cromatográficos de análise. Métodos Cromatográficos de Análise. Rima (2004).
18. Jerz, G., Murillo-Velásquez, J.A., Skrjabin, I., Gök, R., Winterhalter, P.; Anacardic acid profiling in cashew nuts by direct coupling of preparative high-speed countercurrent chromatography and mass spectrometry (prep HSCCC-ESI-/APCI-MS/MS). In *Recent Advances in the Analysis of Food and Flavors*. ACS Publications, Washington, DC, (2012), pp. 145–165.
19. Erşan, S. Güçlü Üstündağ, O. Carle, R. Schweiggert, R.M.; Identification of phenolic compounds in red and green pistachio (*Pistacia vera* L.) hulls (exo- and mesocarp) by HPLC-DAD-ESI-(HR)-MS n; *Journal of Agricultural and Food Chemistry*, 2016; 64(26): 5334–5344
20. Vitolo, M.; Purificação de produtos biotecnológicos; *Revista Brasileira de Ciências Farmacêuticas*, (2005); 41(1): 120–120.
21. Snyder, L.R., Kirkland, J.J., Glajch, J.L.; *Practical HPLC Method Development*. John Wiley & Sons, New York, NY, (2012).
22. Han, S., Liang, C., Yu, H., Qiao, J., Ge, X., Lian, H.; Influence of n-octanol in mobile phase on QSRRs of lipophilicity and retention mechanism of acidic and basic compounds in RP-HPLC; *Royal Society of Chemistry Advances*, (2015); 5(36): 28840–28847.
23. Madureira, T.V., Rocha, M.J., Cass, Q.B., Tiritan, M.E.; Development and optimization of a HPLC-DAD method for the determination of diverse pharmaceuticals in estuarine surface waters; *Journal of Chromatographic Science*, (2010); 48(3): 176–182.
24. Andrisano, V., Makamba, H., Bovina, E., Cavrini, V., Zappoli, S.; HPLC analysis of basic organic compounds in a multi-component ion-interaction system: a mechanistic study; *Chromatographia*, (1998); 47(9): 493–500.
25. Lu, W., Niu, Y., Yang, H., Sheng, Y., Shi, H., Yu, L.L.; Simultaneous HPLC quantification of five major triterpene alcohol and sterol ferulates in rice bran oil using a single reference standard; *Food Chemistry*, (2014); 148: 329–334.
26. Klimczak, I., Gliszczyńska-Świgło, A.; Comparison of UPLC and HPLC methods for determination of vitamin C; *Food Chemistry*, (2015); 175: 100–105.
27. Padilha, C.V.S., Miskinis, G.A., de Souza, M.E.A.O., Pereira, G.E., de Oliveira, D., Bordignon-Luiz, M.T., *et al.*; Rapid determination of flavonoids and phenolic acids in grape juices and wines by RP-HPLC/DAD: method validation and characterization of commercial products of the new Brazilian varieties of grape; *Food Chemistry*, (2017); 228: 106–115.
28. Yuliana, M., Nguyen-Thi, B.T., Faika, S., Huynh, L.H., Soetaredjo, F.E., Ju, Y.; Separation and purification of cardol, cardanol and anacardic acid from cashew (*Anacardium occidentale* L.) nut-shell liquid using a simple two-step column chromatography; *Journal of the Taiwan Institute of Chemical Engineers*, (2014); 45(5): 2187–2193.
29. Praveen Kumar, S.N., Bhadregowda, D.G., Vathsala, D.C., Mantelingu, K., Rangappa, K.S.; Development and validation of a normal phase HPLC method for separation of anacardic acid isomers in cashew nut shell liquid; *Journal of Chemical and Pharmaceutical Research*, (2013); 5 (5): 369–373.
30. Wang, R., Kobayashi, Y., Lin, Y., Rauwald, H.W., Yao, J., Fang, L., *et al.*; HPLC quantification of all five ginkgolic acid derivatives in ginkgo biloba extracts using 13: 0 ginkgolic acid as a single marker compound; *Planta Medica*, (2015); 81(01): 71–78.