Food Chemistry 172 (2015) 725–730

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Validation of HPLC and CE methods for determination of organic acids in sour cassava starch wastewater

Ana Carolina Moura de Sena Aquino, Mônia Stremel Azevedo, Deise Helena Baggio Ribeiro, Ana Carolina Oliveira Costa, Edna Regina Amante *

Federal University of Santa Catarina, Department of Food Science and Technology, Rodovia Admar Gonzaga 1.346, 88034-001 Florianopolis, SC, Brazil

ARTICLE INFO

Article history:
Received 12 October 2013
Received in revised form 14 April 2014
Accepted 10 September 2014
Available online 2 October 2014

Keywords:
Organic acids
Liquid chromatography
Capillary electrophoresis
Sour cassava starch
Fermentation
Method validation

ABSTRACT

Fast and efficient analytical methods to determine the concentrations of lactic, acetic, propionic and butyric acids in sour cassava starch wastewater using reversed-phase high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), were developed and validated. Good linearity ($R^2 > 0.999$) and significance with $F > 25{,}000$ for all acids was showed. The matrix effect was not detected. The coefficient of variation values indicated good repeatability. The limits of detection (LOD) ranged from 1.0 to 3.7 and 2.0 to 3.0, and the limits of quantification (LOQ) from 3.1 to 12.2, and 8.0 to 12.5 mg/L for HPLC and CE, respectively. The quantification of the samples did not reveal significant differences between the methods for all compounds analyzed. However, the benefits of CE in relation to HPLC, such as lower costs and less waste generation, along with shorter analysis times, need to be taken into consideration.

1. Introduction

Cassava (*Manihot esculenta* Crantz) is widely used in human and animal nutrition and also as a raw material for various industrialised products, of which the most important are the cassava flour, cassava starch and sour cassava starch (Avancini et al., 2007). Subjecting cassava starch to a natural fermentation process, varying the conditions such as processing time and location, or to the action of microbial enzymes, promotes the formation of organic acids, yielding the fermented starch, commonly known as sour cassava starch.

During fermentation a diverse microflora develops which is responsible for the production of amylolytic enzymes and organic acids which attack the starch granules (Cereda, 1987), resulting in small areas of corrosion which, together with the other steps of the process, contribute to the functional properties of sour cassava starch as an important expansion agent during baking.

Some studies have identified organic acids, such as lactic, acetic, propionic and butyric acids, in samples of sour cassava starch (Demiate, Senger, Vogler, Cereda, & Wosiacki, 1997; Demiate, Souza, Pugsley, Cereda, & Wosiachi, 1997), and quality differences between the samples were observed in relation to the geographical area of study, which drew attention to the importance of determining the profile of these organic acids.

The fermentation process is of the submerged type and usually occurs with a superficial water layer of 20 cm, which is subsequently considered as an industrial effluent, and thus requires characterisation. According to a study by Avancini et al. (2007), the supernatant water from the fermentation of cassava starch has no toxicity; however, there are few reports available on the composition of the organic acids in these wastewaters.

Alternatives for the use of wastewater from the cassava industry are needed to reduce environmental pollution (Damasceno, Cereda, Pastore, & Oliveira, 2003) and also to contribute to the generation of new income alternatives for producers. As an example, the acids produced during the fermentation of cassava starch are solubilised in the supernatant water, and these are not recovered as products, being considered pollutant organic load in the final effluent.

The techniques used for the identification and quantification of organic acids in other fermented products, such as wine, and wastewaters include liquid chromatography, gas chromatography and capillary electrophoresis (Cruwys, Dindsdale, Hawkes, & Hawkes, 2002; Destendau et al., 2005; Esteves, Lima, Lima, & Duarte, 2004; Kritsunankul, Pramote, & Jakmunee, 2009; Peres et al., 2009; Rizzon & Sganzerla, 2007; Zheng et al., 2009; Ábalos, Bayona, & Pawliszyn, 2000). As noted by several authors, HPLC is

* Corresponding author.
E-mail addresses: carolsena@ig.com.br (A.C.M. de Sena Aquino), moazevedo@gmail.com (M.S. Azevedo), deise@cca.ufsc.br (D.H.B. Ribeiro), ana.costa@ufsc.br (A.C.O. Costa), eamante@cca.ufsc.br (E.R. Amante).

http://dx.doi.org/10.1016/j.foodchem.2014.09.142
0308-8146/

the most widely used technique for the individual identification of these compounds due to its simplicity and precision, as well as the ease of sample preparation which involves only dilution and filtration steps. However, these features are also associated with CE.

In this study, the techniques of reversed-phase HPLC and CE, both with diode array detection (DAD) were evaluated and compared for the determination of organic acids in cassava starch fermentation wastewaters, aiming to contribute to future applications of this waste product which is currently considered to be an agroindustrial residue.

2. Material and methods

2.1. Samples

The supernatant water sample used for the validation of the method was derived from the natural fermentation of cassava starch at room temperature (ranging from 21.5 to 31.0 °C) for 32 days, when it reached the plateau acidity of 2.0 mL of NaOH 0.1 mol/L for each aliquot of 10 mL (Marcon et al., 2006). The sample was kept frozen until the time of analysis. Due to the impossibility of obtaining samples of free organic acids and the high concentration of acetic and butyric acids in the sample, a 50-fold dilution of the sample was obtained with ultrapure water for the evaluation of the precision and accuracy validation parameters, while for lactic and propionic acids the dilution was 2-fold.

Samples of sour cassava starch wastewater were used for the quantification. The fermentation was carried out by two methods: one traditional and one modified (Marcon et al., 2006), between November 2011 and February 2012, in ten 20 L capacity high-density polyethylene tanks. To each tank 10 L of water and 2.5 kg of commercial cassava starch previously homogenised in 1:4 (w/v) (starch/water) (traditional method) were added. To half of the tanks (five), 0.5% glucose corn syrup (Mix, São Paulo, Brazil) (modified method) was added together with the cassava starch suspension at the beginning of the process.

The fermentation process was monitored daily and when the fermentation water reached an acidity plateau of 2.0 mL of NaOH 0.1 mol.L^{-1}/10 mL (19 and 32 days for the modified and the traditional fermentation methods, respectively), the first water sample was collected, and samples were then collected every 15 days until the fermentation was completed 60 days after reaching the acidity plateau (the time commonly employed by sour cassava starch producers in southern Brazil for the removal of the product from the tanks is 60–90 days). After this period, the water from the fermentation process was drained and frozen on circular stainless steel trays in a plate freezer and stored at −18 °C until analysis.

Since organic acids are solubilised in sour cassava starch wastewater, the samples were filtered through a 0.22 μm polytetrafluoroethylene membrane (Allcrom, São Paulo, Brazil) before injection. For the quantification by HPLC and CE the samples were diluted as required to achieve the detection range of the method.

2.2. High performance liquid chromatography

2.2.1. Reagents and solutions

All chemicals used in the experiments were of analytical reagent grade and ultrapure water with a resistivity of 18.2 MΩ cm obtained from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all solutions. Lactic, butyric, acetic and propionic acids were obtained from Fluka (São Paulo, Brazil). Methanol was supplied by J.T. Baker (Mallinckrodt, Phillipsburg, NJ, USA) and potassium monobasic phosphate by Proquimios (Rio de Janeiro, Brazil).

Standard stock solutions containing 800 mg/L of the organic acids were prepared in ultrapure water. Calibration solutions were prepared by diluting the stock solutions with ultrapure water and the final concentrations of the working solutions are given in Table 1.

The mobile phase consisted of a mixture of 0.02 mol/L KH_2PO_4 buffer solution (pH = 2.88 ± 0.02 adjusted with *o*-phosphoric acid) and 2% methanol, filtered through a 0.45 μm cellulose acetate membrane (Advantec MFS, Tokyo, Japan), for lactic, acetic and propionic acids (Zotou, Loukou, & Karava, 2004). For butyric acid, a mixture of 0.02 mol/L KH_2PO_4 buffer solution (pH = 2.88 ± 0.02 adjusted with *o*-phosphoric acid) and 40% methanol, filtered through a 0.45 μm polytetrafluoroethylene membrane (Allcrom, São Paulo, Brazil) was used as the mobile phase.

2.2.2. Liquid chromatography system

The separation module used was equipped with a quaternary pump and degasser (model LC-20AT), an autosampler (model SIL-10A) adjusted to 20 μL volume injection, a diode array detector (model SPD-M20A) with a working wavelength range of 190–230 nm, controlled by a workstation (model CBM-20A) with data acquisition system LC Solutions software, all manufactured by Shimadzu Corporation (Kyoto, Japan). For the stationary phase a C18 column, Hichrom (150 × 4.6 mm, Berkshire, UK) was used. An isocratic elution procedure was applied to the mobile phases with a flow rate of 0.6 mL/min. The peaks were identified by their retention times, comparing the UV–visible spectra and spiking with standards. A wavelength of 220 nm was selected for the quantification.

2.3. Capillary electrophoresis

2.3.1. Reagents and solutions

All chemicals used in the experiments were of analytical reagent grade and ultrapure water with a resistivity of 18.2 MΩ cm was obtained from a Milli-Q system (Millipore, Bedford, MA, USA) and used to prepare all solutions. Benzoic, acetic, lactic, propionic and trifluoroacetic acids (as internal standard, I.S.), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-TRIS) and cetyltrimethylammonium bromide (CTAB) were obtained from Sigma–Aldrich (São Paulo, Brazil). Butyric acid was obtained from Fluka (São Paulo, Brazil). Standard stock solutions containing 1000 mg/L of the organic acids were prepared in ultrapure water. Calibration solutions were prepared by diluting the stock solutions with ultrapure water and the final concentrations are given in Table 1. Before injection into the CE equipment, these solutions were diluted with I.S. solution (9:1, v/v, sample:I.S. at 250 mg/L).

2.3.2. Capillary electrophoresis system

CE assays were conducted on a capillary electrophoresis system (model 7100, Agilent Technologies, Palo Alto, California, USA), equipped with a diode array detector (set at 234 nm; indirect detection, with a reference at 360 nm for peak inversion), a temperature-control device (maintained at 25 °C), and data acquisition and treatment software supplied by the manufacturer (HP ChemStation, rev. A.06.01). A fused-silica capillary (Polymicro Technologies, Phoenix, Azusa, USA) with dimensions of 48.5 cm total length, 40 cm effective length and 50 μm inner diameter was employed in the separations. The activation of the capillary surface by dissociation of the silanol groups was performed by flushing with 1 mol/L NaOH for 20 min, deionized water for 20 min and electrolyte solution for 10 min. In between runs, the capillary was flushed with background electrolyte (BGE) solution for 1 min. The BGE used to determine the organic acids was composed of 20 mmol/L benzoic acid, 25 mmol/L BIS-TRIS, 0.2 mmol/L CTAB at pH 5.8. The standards and samples were injected using a hydrodynamic pressure

Table 1
Concentrations of acetic, lactic, propionic and butyric acids employed for the calibration curves – CE and HPLC.

Method	Compounds	Level (mg/L)						
		1	2	3	4	5	6	7
CE	Acetic	8.0	12.5	25.0	50.0	100.0	200.0	400.0
	Lactic	12.5	25.0	50.0	100.0	200.0	400.0	–
	Propionic	8.0	12.5	25.0	50.0	100.0	200.0	400.0
	Butyric	10.0	12.5	25.0	50.0	100.0	200.0	400.0
HPLC	Acetic	6.5	12.5	25.0	50.0	100.0	200.0	400.0
	Lactic	6.3	12.5	25.0	50.0	100.0	200.0	400.0
	Propionic	12.2	25.0	50.0	100.0	200.0	400.0	–
	Butyric	3.1	12.5	25.0	50.0	100.0	200.0	400.0

of 50 mbar for 3 s. The separation voltage applied was 30 kV, with negative polarity on the injection side.

2.4. Evaluation of the intra-laboratory validation parameters

The parameters evaluated according to ANVISA – Agência Nacional de Vigilância Sanitária (2003), employing assays with standard solutions, blank samples and spiked samples, were calibration curve linearity, matrix effects, precision, accuracy, recovery, and limits of detection and quantification. The linearity was assessed by obtaining three calibration curves for organic acids with three independent replicates (for levels see Table 1). Blanks were also prepared, in triplicate, for each curve to set zero on the equipment. *F*-tests were carried out to check the fitting with the model through the evaluation of the regression (Draper & Smith, 1981).

Matrix effects, for both methods, were verified by applying the standard additions method. Matrix-matched curves were prepared according to the procedure used to assess the linearity for the matrix free of organic acids. Three independent replicates were run in a random order on the same day. Blanks were also prepared to set zero on the equipment. The values for the slope and intercept, and the respective variances, of both curves were calculated by the ordinary least square method (OLSM). The slopes and intercepts obtained for the solvent and matrix-matched calibration functions were compared to verify significant differences applying the *t*-test (Armitage & Berry, 1994). The hypothesis tests were performed at the $\alpha = 0.05$ level.

The precision was determined considering the mean of seven consecutive injections of the standard mixtures of the organic acids at levels of 20, 100 and 200 mg/L using the I.S. ratio and considering the relative standard deviation (RSD%). All analysis was carried out on the same day, by the same analyst, using the same methodology and equipment.

The accuracy was investigated considering the mean of seven consecutive injections of the matrix matched with the standard mixtures of the organic acids at levels of 20, 100 and 200 mg/L with the results expressed in terms of the measured amount of the compounds in relation to the amount added to the matrix. The minimum trueness criteria range was ±20% (ANVISA, 2003).

The LOQ was defined as the lowest concentration at which the method could operate with acceptable precision (signal/noise ratio $\geqslant 10$). The LOD was considered as the lowest concentrations of the organic acids that were detectable in all replicates but not necessarily quantified and distinguished from zero.

2.5. Statistical analysis

The data were analyzed using the software Statistica Version 7.0 (StatSoft, Inc. USA). Normality of the data obtained by HPLC and CE methods was verified by applying the Shapiro–Wilk *W*-test (Royston, 1983; Shapiro & Wilk, 1965). A p value of >0.05 indicates normal distribution. A p value of <0.05 indicates non-normal distribution. In cases where there was significant evidence of non-normality, which can be ascribed to skewness, heavy tails or outliers etc. (Gel, Miao, & Gastwirth, 2007), the non-parametric Mann–Whitney *U*-test was used to identify differences between independent groups of data obtained by HPLC and CE and to determine the statistical significance (Burke, 2001; Mann & Whitney, 1947). Non-parametric tests of location, such as the Mann–Whitney test, can be far more powerful than the *t*-test in the case of certain leptokurtic distributions (Bonett & Seier, 2002).

3. Results and discussion

3.1. High performance liquid chromatography

With a mobile phase solution of 0.02 mol/L KH_2PO_4 buffer solution (pH 2.88 ± 0.02) and 2% methanol using an isocratic elution mode with a flow rate of 0.6 mL/min, the total time of the HPLC analysis was 14 min for lactic, acetic and propionic acids, while for butyric acid with a mixture of 0.02 mol/L KH_2PO_4 buffer solution (pH 2.88 ± 0.02) and 40% methanol, the retention time was 6.6 min. The mobile phases were acidified with phosphoric acid, since the addition of acid to the mobile phase will suppress the ionisation of acidic functional groups and promote greater interaction of the compounds with the stationary phase (Cerqueira et al., 2011).

Figs. 1 and 2 show the chromatograms for each compound, for the 200 mg/L standard mixture, for a blank of the diluted samples and for the diluted samples with the addition of the 200 mg/L standard mixture.

3.2. Capillary electrophoresis

3.2.1. Background electrolyte optimisation

The optimisation of the BGE for the proposed quantification method employing capillary zone electrophoresis (CZE) was

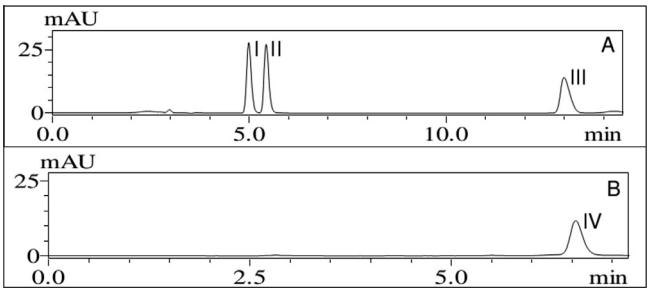

Fig. 1. Chromatograms for the 200 mg/L standard solution of the organic acids (A) I – lactic acid, II – acetic acid, III – propionic acid and (B) IV – butyric acid. Description: This figure show the elution time for the organic acids standard solution, in the concentrations that can be found in the samples. These chromatograms are the base to organic acids determination in the samples and to the adjustment of the equipment and all conditions of it.

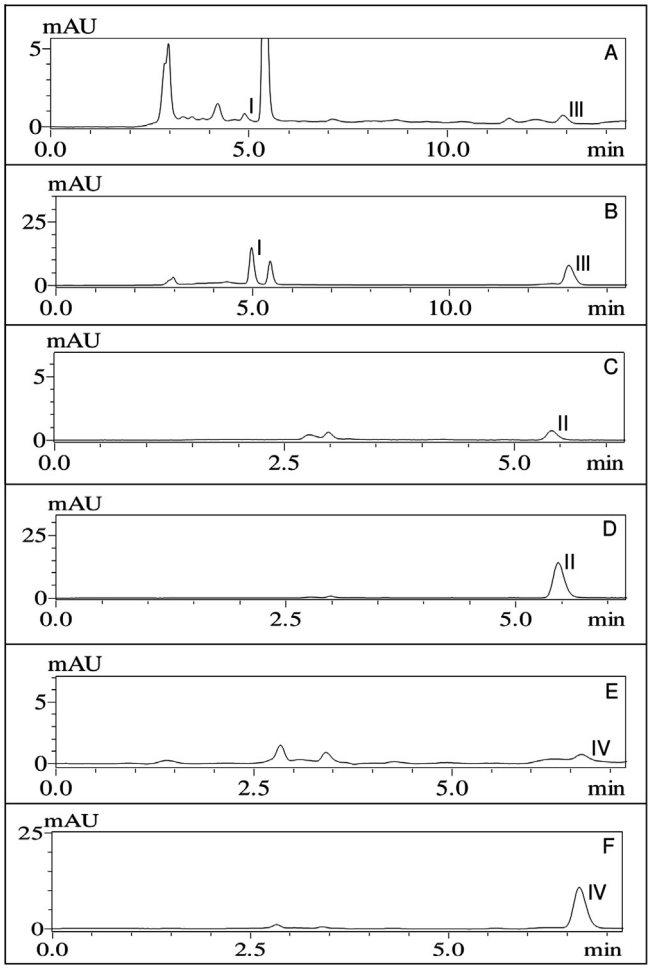

Fig. 2. Chromatograms (A) I – lactic acid and III – propionic acid in the sample with 2-fold dilution, (B) I – lactic acid and III – propionic acid in the sample with 2-fold dilution and the addition of the 200 mg/L standard, (C) II – acetic acid in the samples with 50-fold dilution, (D) II – acetic acid in the sample with 50-fold dilution and the addition of the 200 mg/L standard, (E) IV – butyric acid in the sample with 50-fold dilution and (F) IV – butyric acid in the sample with 50-fold dilution and the addition of the 200 mg/L standard. Description: This figure presents the chromatograms and the respective elution times for the organic acids analyzed in the sour cassava starch wastewater. Where is possible found that each analyzed acid was detected according the conditions established to the standard of respective acids.

performed considering the organic acids found in the samples. To select the optimum separation conditions, data related to the pH, co-ion and counter-ion of the BGE, I.S. and other system parameters of separation were used to plot the effective mobility *versus* pH using the Peakmaster® software (data not show). The pH was selected considering the ionised analytes, and the difference in mobility required for the separation was reached at pH 5.8. The components of the BGE selected to provide this pH condition were benzoic acid as the co-ion and BIS-TRIS as the counter-ion, at concentrations of 20 and 25 mmol/L, respectively. This co-ion was selected because it has chromophore groups in its structure, enabling the indirect detection of analytes that do not absorb in the UV–Vis range. BIS-TRIS was chosen as the counter-ion since it has a pKa of 6.4, a value close to the separation pH, thereby conferring an adequate buffering capacity to this method.

The internal standard selected was trifluoroacetic acid, which has characteristics similar to those of the analytes and was not present in the sample matrix. To promote the separation of organic acids in the shortest possible time, CTAB was used as an electroosmotic flow (EOF) inverter. The simulation of the separation of organic acids was obtained using the Peakmaster® software and a good similarity was observed between the simulated and experimental electropherograms for the standards, verifying that the separation conditions chosen were appropriate (data not show). The experimental electropherograms obtained employing the optimised conditions are shown in Fig. 3.

3.3. Evaluation of the intra-laboratory validation parameters

As can be seen in Table 2, for both CE and HPLC, the calibration curves show good linearity (coefficient of determination >0.999) and the linear models showed significance with $F > 25{,}000$ for lactic, acetic, propionic and butyric acids.

No matrix effects were detected in the range studied. Lack of significance ($p > 0.05$) was observed when the slope and the intercept of the solvent curve were compared with those from the matrix-matched curve applying the *t*-test, since the variances were considered homogenous when compared using the *F*-test ($p > 0.05$). Good repeatability was observed since the values for the coefficient of variation for the repeatability ranged from 0.10% to 1.15% and 0.89% to 4.87% for the HPLC and CE methods, respectively. These values are considered appropriate according to Brazilian legislation, which establishes a precision of up to 5% (ANVISA, 2003).

The accuracy values ranged between 92.6% and 103.8% for HPLC and between 87.9% and 102.9% for CE. These values are in agreement with the officially acceptable range of 80–120% (ANVISA,

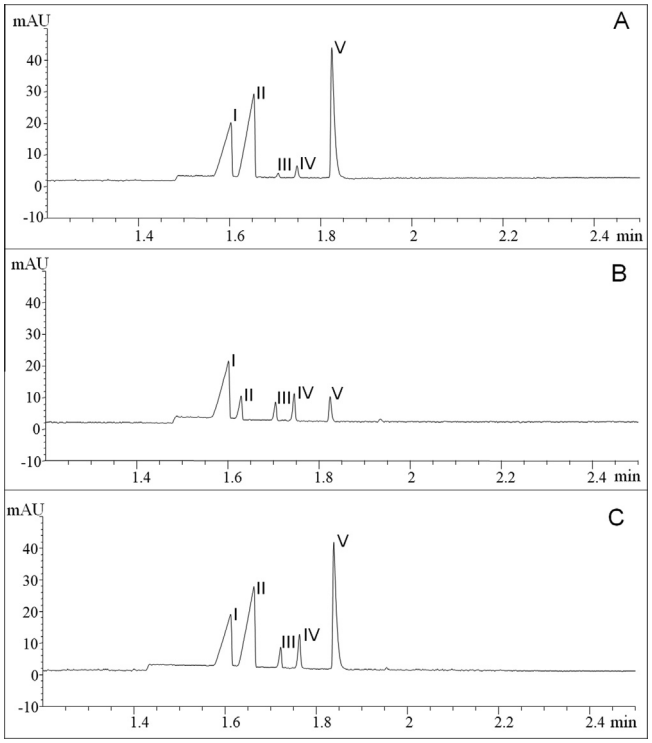

Fig. 3. Electropherograms for (A) Blank sample with 2-fold dilution; (B) 25 mg/L standard mixture; (C) Blank sample with 2-fold dilution and 25 mg/L standard mixture of organic acids; I: 250 mg/L Trifluoroacetic acid (I.S.); II: acetic acid; III: lactic acid; IV: propionic acid; V: butyric acid. Description: This figure is showing the result of the electrophoresis capilar analyses for the organic acids. The first (A) electropherogram show the blank sample that means the response of the signal for the samples with the double of dilution, to safe that if the compounds in analysis are concentrated and the method is efficient to this sample. The second mean the signals of a standard mixture of all acids being studied, including the trifluoroacetic acid (internal standard), as a references for the effective sample analysis. Finally, in the section (C) of the figure, the blank of the samples with the double of dilution and the standard mixture signals.

Table 2
Analytical performance for HPLC and CE methods.

Parameter	*n*	Lactic acid		Acetic acid		Propionic acid		Butyric acid	
		HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE
[a]Precision (% RSD at 20 mg/L)	7	0.63	3.51	0.57	4.87	1.15	2.66	1.05	2.77
[a]Precision (% RSD at 100 mg/L)	7	0.28	1.09	0.29	2.89	0.65	0.89	0.38	2.64
[a]Precision (% RSD at 200 mg/L)	7	0.34	1.39	0.27	2.72	0.41	1.37	0.10	1.91
[b]Accuracy (% at 20 mg/L)	7	99.89 ± 0.63	95.57 ± 3.35	101.93 ± 0.58	98.42 ± 4.79	103.77 ± 1.20	93.96 ± 2.49	98.22 ± 1.04	102.89 ± 2.85
[b]Accuracy (% at 100 mg/L)	7	102.32 ± 0.29	97.62 ± 1.06	100.14 ± 0.29	93.15 ± 2.69	102.31 ± 0.67	94.95 ± 0.84	99.24 ± 0.38	97.15 ± 2.57
[b]Accuracy (% at 200 mg/L)	7	101.47 ± 0.35	99.9 ± 1.39	100.26 ± 0.27	97.27 ± 2.64	97.94 ± 0.41	87.87 ± 1.34	92.64 ± 0.10	101.94 ± 1.95
Linearity – linear range (mg/L)		6.3–400.0	12.5–400.0	6.5–400.0	8.0–400.0	12.2–400.0	8.0–400.0	3.10–400.0	10.0–400.0
Linearity – slope		582.5	0.0041	587.9	0.0069	596.3	0.0063	629.4	0.0059
Linearity – intercept		211.4	0.0012	339.7	0.0165	636.5	0.0031	1084.4	0.0008
Linearity – Coefficient of determination, R^2		0.9999	0.9995	0.9999	0.9996	0.9999	0.9996	0.9999	0.9995
LOD (mg/L)		1.9	3.0	1.9	2.0	3.7	3.0	1.0	3.0
LOQ (mg/L)		6.3	12.5	6.5	8.0	12.2	8.0	3.1	10.0
[c]*F*		1.01×10^6	50,864	4.99×10^5	25,007	2.44×10^6	36,103	1.46×10^5	73,809

[a] Coefficient of variation.
[b] Mean ± standard deviation.
[c] ANOVA.

2003). The LOD values were 1.9 and 3.0 mg/L for lactic acid, 1.9 and 2.0 mg/L for acetic acid, 3.7 and 3.0 mg/L for propionic acid and 1.0 and 3.0 mg/L for butyric acid, for the HPLC and CE methods, respectively. The LOQ values were 6.3 and 12.5 mg/L for lactic acid, 6.5 and 8.0 mg/L for acetic acid, 12.2 and 8.0 mg/L for propionic acid, 3.1 and 10.0 mg/L for butyric acid, for the HPLC and CE methods, respectively. For the applicability of the method, these values are adequate since the concentrations of organic acids exceeded these amounts in the samples analyzed.

3.4. Sour cassava starch wastewater analysis

In order to apply the optimised methods to real samples, the organic acids contents of the wastewater generated in the production of sour cassava starch were determined applying the HPLC and CE methods and the samples were prepared in triplicate. The results ranged from 12.76 to 126.55 mg/L for lactic acid, 398.91 to 1180.32 mg/L for acetic acid, 57.67 to 201.96 mg/L for propionic acid and 359.71 to 1921.76 mg/L for butyric acid applying the CE method, while the results ranged from 16.68 to 124.17 mg/L for lactic acid, 469.58 to 1336.03 mg/L for acetic acid, 56.72 to 201.98 mg/L for propionic acid and 387.29 to 1979.65 mg/L for butyric acid applying the HPLC method.

The non-normality of the data obtained was verified using the Shapiro–Wilk *W*-test for acetic, butyric, lactic and propionic acids ($p < 0.05$). The data were then compared using the non-parametric Mann–Whitney (rank sum) *U*-test which indicated no significant difference between the data obtained by the HPLC and CE methods for all compounds analyzed ($p > 0.05$) (Table 2). The concentrations (mg/L) of organic acids found in the samples applying the HPLC and CE methods are reported in Table 3.

There is a predominance of acetic and butyric acids in all samples, in contrast to the observations noted in the majority of published studies on the fermentation of cassava starch as well as the quantification of the organic acids in sour cassava starch (Carvalho, Canhos, Ribeiro, & Carvalho, 1996; Cárdenas & Buckle, 1980; Demiate, Barana, Cereda, & Wosiacki, 1999; Parada, Fabrizio, & Martinez, 1996; Silveira, Carvalho, Padua, & Dionizio, 2003), which indicated that lactic fermentation predominated.

The fermentations in this study were conducted in the laboratory in the summer of 2011/2012 and the temperatures of the environment and tanks reached 35 °C during the fermentation process.

Table 3
[a]Concentration (mg/L) of organic acids in the sour cassava starch wastewater determined by HPLC and CE methods, at different times.

Samples		Lactic acid		Acetic acid		Propionic acid		Butyric acid	
Sour cassava starch wastewater	Time (days)	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE
Traditional	32	16.68 ± 0.41	17.08 ± 0.24	469.58 ± 3.18	398.91 ± 4.17	61.49 ± 2.08	57.67 ± 0.77	387.29 ± 0.82	359.71 ± 3.87
	46	35.81 ± 0.85	32.59 ± 1.67	788.03 ± 1.93	717.92 ± 9.55	56.72 ± 0.57	59.67 ± 0.66	609.69 ± 3.41	677.57 ± 13.30
	61	121,96 ± 0.37	117.73 ± 3.85	1336.03 ± 3.13	1178.32 ± 11.80	74.15 ± 0.30	74.77 ± 0.80	1374.51 ± 5.68	1132.87 ± 11.10
	74	20.50 ± 0.77	21.28 ± 0.80	833.01 ± 0.49	776.60 ± 7.55	121.72 ± 2.59	119.18 ± 2.25	707.46 ± 3.06	617.54 ± 2.45
	85	124.17 ± 0.26	126.55 ± 3.13	1045.75 ± 3.83	1180.32 ± 10.70	67.95 ± 0.25	71.50 ± 1.95	1118.38 ± 12.65	1072.71 ± 10.50
Modified	19	18.56 ± 0.65	15.73 ± 0.61	634.26 ± 5.58	581.10 ± 10.95	107.71 ± 1.57	111.56 ± 1.76	933.59 ± 1.57	956.09 ± 16.82
	33	15.39 ± 0.31	13.20 ± 2.33	638.91 ± 8.59	601.95 ± 4.89	120.50 ± 2.32	119.71 ± 4.46	947.16 ± 4.39	1118.56 ± 5.87
	48	60.07 ± 0.91	57.23 ± 0.71	1150.06 ± 0.13	1059.38 ± 10.40	153.64 ± 0.28	157.37 ± 1.07	962.98 ± 5.50	950.53 ± 1.87
	62	24.26 ± 0.68	23.46 ± 2.24	1263.78 ± 7.61	1011.04 ± 22.12	179.28 ± 2.85	181.44 ± 1.39	1184.20 ± 5.35	966.57 ± 20.06
	75	14.39 ± 1.17	12.76 ± 1.15	628.49 ± 1.49	625.16 ± 10.10	201.98 ± 2.10	201.96 ± 4.54	1979.65 ± 1.21	1921.76 ± 31.10
Non-normality test (Shapiro–Wilk *W*) – *p* value		0.00000		0.00120		0.00008		0.00008	
Non-parametric test (Mann–Whitney *U*) – *p* value		0.6361		0.2089		0.8245		0.7338	

[a] mean ± standard deviation ($n = 3$).

Demiate et al. (1999) studied 29 samples of fermented cassava starch in the south and southeast regions of Brazil, purchased directly from factories or commercial outlets. The authors found concentrations of between 120 and 830 mg/kg of lactic acid, ND (not detected) and 680 mg/kg of acetic acid, ND and 130 mg/kg of propionic acid, and ND and 570 mg/kg of butyric acid, which are lower (with the exception of lactic acid), than the values obtained for the sour cassava starch wastewaters analyzed in this study.

Reginatto et al. (2009) performed cassava starch fermentation with modifications, adding glucose as a carbon source and ammonium as a nitrogen source to verify their effect on the production of organic acids and on the characteristics of the sour cassava starch. In the fermentation modified with glucose carried out for 45 days, the concentrations found in the wastewater acid varied between ND and 650 mg/L for lactic and acetic acids, with around 200 mg/L of propionic acid, while acid butyric acid was not detected.

The results presented in this study indicate that these two analytical techniques are appropriate for the determination of organic acids produced in the fermentation of cassava starch, which are emerging as new raw materials obtained from the production of sour cassava starch. In the future, these methods may represent important tools to define the use of these waters. The techniques described herein are simple, fast and effective for this type of analysis.

4. Conclusion

It is importance to monitor the concentrations of organic acids present during the cassava starch fermentation process given the need to add value to the wastewater generated during sour cassava starch production. The HPLC and CE methods described in this paper were validated for the identification and quantification of lactic, acetic, propionic and butyric acids in a sample of this type of wastewater. There were no significant differences between the methods in terms of analytical performance, considering the compounds analyzed. However, some benefits of CE compared with HPLC, such as lower cost, waste generation and analysis time (<2 min), should be taken into consideration prior to their application.

Acknowledgements

The authors are grateful to the Brazilian governmental agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for their financial support and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for scholarships.

References

Ábalos, M., Bayona, J. M., & Pawliszyn, J. (2000). Development of a headspace solid-phase microextraction procedure for the determination of free volatile fatty acids in wastewaters. *Journal of Chromatography A, 873*, 107–115.

ANVISA – Agência Nacional de Vigilância Sanitária (2003). Resolução RDC n 899 de 29 de maio de 2003. Brasília: Diário Oficial da União 2003 (31.03.03).

Armitage, P., & Berry, G. (1994). *statistical methods in medical research* (3rd ed.). Oxford: Blackwell.

Avancini, S. R. P., Faccin, G. L., Tramonte, R., Rovaris, A. A., Podestá, R., Souza, N. M. A., et al. (2007). Cassava starch fermentation wastewater: Characterization and preliminary toxicological studies. *Food and Chemical Toxicology, 45*, 2273–2278.

Bonett, D. G., & Seier, E. (2002). A test of normality with high uniform power. *Computational Statistics & Data Analysis, 40*, 435–445.

Burke, S. (2001). Missing values, outliers, robust statistics and non-parametric methods. *Statistics and Data Analysis*. LCGC Europe online Supplement, 19–24.

Cárdenas, O. S., & Buckle, T. S. (1980). Sour cassava starch production: a preliminary study. *Journal of Food Science, 45*, 1509–1512.

Carvalho, E. P., Canhos, V. P., Ribeiro, V. E., & Carvalho, H. P. (1996). Polvilho Azedo: Aspectos físicos, químicos e microbiológicos. *Pesquisa Agropecuária Brasileira, 31*(2), 129–137.

Cereda, M. P. (1987). Tecnologia e qualidade do polvilho azedo. *Informe Agropecuário, 13*(145), 63–68.

Cerqueira, M. B. R., Dias, A. N., Caldas, S. S., Santana, F. B., D'oca, M. G. M., & Primel, E. G. (2011). Validação de método para determinação de ácidos orgânicos voláteis em efluentes de reatores anaeróbicos empregando cromatografia líquida. *Química Nova, 34*(1), 156–159.

Cruwys, J. A., Dindsdale, R. M., Hawkes, F. R., & Hawkes, D. L. (2002). Development of a static headspace gas chromatographic procedure for the routine analysis of volatile fatty acids in wastewaters. *Journal of Chromatography A, 945*, 195–209.

Damasceno, S., Cereda, M. P., Pastore, G. M., & Oliveira, J. G. (2003). Production of volatile compounds by *Geotrichum fragrans* using cassava wastewater as substrate. *Process Biochemistry, 39*(4), 411–414.

Demiate, I. M., Senger, S. A., Vogler, Z., Cereda, M. P., & Wosiacki, G. (1997a). Características de qualidade de amostras de polvilho azedo produzido ou comercializado no Estado do Paraná. *Arquivos de Biologia e Tecnologia, 40*(2), 331–349.

Demiate, I. M., Souza, T. O., Pugsley, S., Cereda, M. P., & Wosiacki, G. (1997b). Características de qualidade de amostras de polvilho azedo. *Agropecuária Catarinense, 10*(4), 51–56.

Demiate, I. M., Barana, A. C., Cereda, M. P., & Wosiacki, G. (1999). Organic acid profile of commercial sour cassava starch. *Ciência e Tecnologia de Alimentos, 19*(1), 131–135.

Destendau, E., Vial, J., Jardy, A., Hennion, M. C., Bonnet, D., & Lancelin, P. (2005). Development and validation of a reserved-phase liquid chromatography method for the quantitative determination of carboxylic acids in industrial reaction mixtures. *Journal of Chromatography A, 1088*, 49–56.

Draper, N. R., & Smith, H. (1981). *Applied regression analysis* (2nd ed.). New York: John Wiley.

Esteves, V. I., Lima, S. S. F., Lima, D. L. D., & Duarte, A. C. (2004). Using capillary electrophoresis for the determination of organic acids in port wine. *Analytica Chimica Acta, 5131*, 163–167.

Gel, Y. R., Miao, W., & Gastwirth, J. L. (2007). Robust directed tests of normality against heavy-tailed alternatives. *Computational Statistics & Data Analysis, 51*, 2734–2746.

Kritsunankul, O., Pramote, B., & Jakmunee, J. (2009). Flow injection on-line dialysis coupled to high performance liquid chromatography for the determination of some organic acids in wine. *Talanta, 79*, 1042–1049.

Mann, H. B., & Whitney, D. R. (1947). On a test whether one of two random variables is stochastically larger than the other. *The Annals of Mathematic Statistics, 18*(1), 50–60.

Marcon, M. J. A., Vieira, M. A., Santos, K., De Simas, K. N., Amboni, R. D. M. C., & Amante, E. R. (2006). *Journal of Food Process Engineering, 29*, 362–372.

Parada, J. L., Fabrizio, S. V., & Martinez, A. (1996). Microbiological and technological aspects of cassava-starch fermentation. *World Journal of Microbiology & Biotechnology, 12*(1), 53–56.

Peres, R. G., Moraes, E. P., Micke, G. A., Tonin, F. G., Tavares, M. F. M., & Rodriguez-Amaya, D. B. (2009). Rapid method for the determination of organic acids in wine by capillary electrophoresis with indirect UV detection. *Food Control, 20*, 548–552.

Reginatto, V., Kurtz, D., Marcon, M. J. A., Xavier, J. J. M., Scussel, V. M., & Amante, E. R. (2009). Modificação do processo de produção de polvilho azedo visando o aumento na concentração de ácidos orgânicos na água residuária. 2nd International Workshop Advances in Cleaner Production.

Rizzon, L. A., & Sganzerla, V. M. A. (2007). Ácidos tartárico e málico no mosto de uva em Bento Gonçalves. *Ciência Rural, 37*, 911–914.

Royston, J. F. (1983). Some techniques for assessing multivariate normality based on the Shapiro-Wilk W. *Applied Statistics, 32*, 121–133.

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika, 52*, 591–611.

Silveira, I. A., Carvalho, E. P., Padua, I. P. M., & Dionizio, F. L. (2003). Isolamento e caracterização da microbiota ácido lática envolvida no processo fermentativo para produção de polvilho azedo – parte II. *Pro Homine, 2*(2), 7–15.

Zheng, Y. J., Duan, Y. T., Zhang, Y. F., Pan, Q. H., Li, J. M., & Huang, W. D. (2009). Determination of organic acids in red wine and must on only one RP-LC-Column directly after sample dilution and filtration. *Chromatographia, 69*, 1391–1395.

Zotou, A., Loukou, Z., & Karava, O. (2004). Method development for the determination of seven organic acids in wines by reversed-phase high performance liquid chromatography. *Chromatographia, 60*, 39–44.