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Short communication

Capillary zone electrophoresis for separation and analysis of hydroxycitric acid and hydroxycitric acid lactone: Application to herbal products of *Garcinia atroviridis* Griff

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Abstract

Capillary zone electrophoresis (CZE) was developed for quantitative determination of hydroxycitric acid and hydroxycitric acid lactone in herbal products of *Garcinia atroviridis* Griff. Resolution optimization was investigated by varying type, concentration and pH of buffers. Using the pH 9.2 buffer containing 30 mM $Na_2B_4O_7$, 90 mM NaH_2PO_4 and 0.5 mM tetradecyltrimethyl ammonium bromide, baseline resolution ($R_s > 1.5$) was found for all analytes. Advantages of the developed CZE method include simple sample preparation, fast analysis time within 5 min and high accuracy and precision.

Keywords: Carboxylic acids; Capillary electrophoresis; *Garcinia atroviridis* Griff; Hydroxycitric acid; Hydroxycarboxylic acid

1. Introduction

Hydroxycitric acid (1,2-dihydroxypropane-1,2,3-tricarboxylic acid, HCA) is a tricarboxylic acid, and its structure is similar to citric acid (CA) as shown in Fig. 1. Recent reviews of chemistry, biochemistry, physiological properties and microbial production of HCA have been reported [1,2]. (2*S*,3*S*)-HCA is widely found in the fruit rinds of *Garcinia* genus, including *G. cambogia*, *G. indica* and *G. atroviridis* [1,2], while (2*S*,3*R*)-HCA is enriched in the calyxes of *H. subdariffa* [2], and may be obtained from microbial production [2]. Other HCA stereoisomers (2*R*,3*R*)- and (2*R*,3*S*)-HCA, have not been isolated from natural resources. In preliminary research based on laboratory and animal tests, (2*S*,3*S*)-HCA has been proven to inhibit the function of ATP citrate lyase to convert excess glucose into fat [3]. This indicates that HCA suppresses appetite and induces weight loss [4,5]. Contradictory results for HCA on weight loss in human have been reported [6,7]. The other HCA stereoisomers have no potent of inhibition of ATP citrate lyase [2]. In addition, according to evaluation of the genotoxicity using an *in vivo* micronucleus test [8], (2*S*,3*S*)-HCA has been recently found to preferentially induce micronuclei by increasing the number of MNPCEs (micronucleated polychromatic erythrocytes/1000 polychromatic erythrocytes) and PCE/(PCE + NCE) ratios.

Most of previous works and this work involve HCA in *Garcinia* genus, and therefore, HCA refers to (2*S*,3*S*)-HCA. Weight loss products containing HCA from *G. atroviridis* are widely available in the market in Thailand. The herbal products may contain hydroxycitric acid lactone (HCAL), obtained from the original plant and/or lactonization of HCA during boiling and drying the products [1]. Since HCAL shows less effective inhibition than does (2*S*,3*S*)-HCA [2], quantitative analysis of individual HCA and HCAL in herbal products is important for quality control and effective use. The conventional method used for determination of HCA content involves acid–base titration, which gives total acidity of the extract [9].

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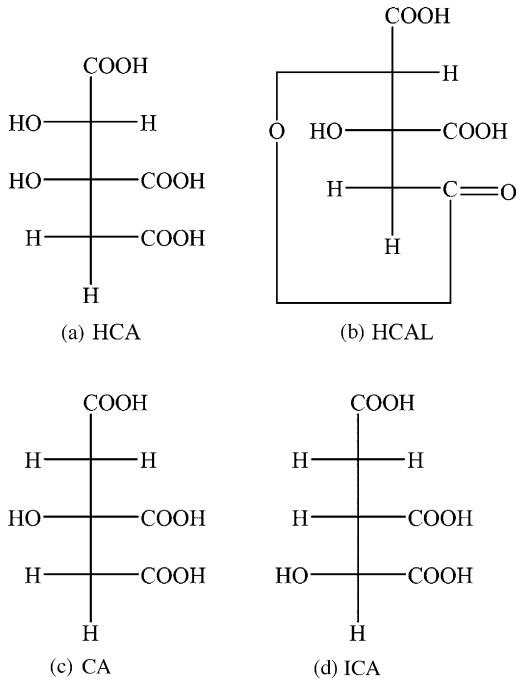

Fig. 1. Structures of carboxylic acids. (a) Hydroxycitric acid (HCA), (b) hydroxycitric acid lactone (HCAL), (c) citric acid (CA) and (d) isocitric acid (ICA).

This method cannot estimate HCA in the products containing HCAL and minor organic acids such as citric acid, tartaric acid and malic acid [1]. High performance liquid chromatography (HPLC) has been reported as a method for determination of HCA, HCAL and other acids in *Garcinia* fruits and products [10], *G. cambogia* fruits and extracts [11,12], and leaves and rinds of *G. indica* [13] and *G. cowa* [14]. However, this HPLC method suffered from poor resolution of some analytes and the low pH mobile phase used. In addition, direct injection of the extract into an HPLC column reduces the column efficiency and shortens the column life [15]. Therefore, sample preparation, such as the aqueous extract through an ODS cartridge, may be needed to remove interfering substances such as pigments and xanthones [15].

Capillary electrophoresis (CE) has been shown to be an excellent method for separation and determination of small organic acids, especially di- and tri-carboxylic acids as well as hydroxycarboxylic acids including citric acid, in samples of fruits, plants and fruit juice [16–21]. CE analysis of these organic acids was performed using basic buffers, such as phosphate [18–20] or borate [22], and direct UV detection. In order to obtain fast analysis of anionic analytes having high electrophoretic mobility, suppressed electroosmotic flow (EOF) using permanently coated capillaries [19,20] or reversed EOF with dynamically coated capillaries was used with the reversed polarity of the applied voltage [21,22]. An alternative way to CE analysis of organic acids was performed using indirect detection with phthalate as UV absorbing background electrolyte (BGE), reversed EOF and reversed polarity [21,23]. EOF can be reversed by addition of cationic surfactants to the buffer, such as cetyltrimethylammonium bromide [18,21,23] and tetradecyltrimethylammonium bromide (TTAB) [21,23]. Separation of carboxylic acids may be improved by adding Mg^{2+} in the buffer to form complexation, resulting in a change in effective electrophoretic mobility of analytes but longer analysis time [22]. Recent reviews, covering the literatures up to 2005 [24] and 2007 [25], have been reported on CE analysis of organic acids including short-chain carboxylic acids.

Up-to-date, CE analysis of HCA and HCAL has not been reported. This work aims to develop capillary zone electrophoresis (CZE) as a method for determination of HCA and HCAL in herbal products of *G. atroviridis*. CZE separation will be carried out using basic buffers such as phosphate, borate and a mixture thereof. Other parameters on separation of analytes will be investigated: concentration and pH of the buffer.

2. Experimental

2.1. Chemicals

(2*S*,3*S*)-Hydroxycitric acid calcium salt ($(C_6H_5O_8)_2Ca_3$) was obtained from Wako Pure Chemical Industries Ltd. (Japan). CA, (+/−)-isocitric acid (ICA), oxalic acid (OA), (+/−)-malic acid (MA) and (2*R*,3*R*)-tartaric acid (TA) were purchased from Sigma–Aldrich (Steinheim, Germany). The following chemicals used were at least the analytical grade and purchased from Fluka (Buchs, Switzerland): $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}12H_2O$ and $Na_2B_4O_7{\cdot}10H_2O$. H_3PO_4 and TTAB were obtained from Sigma–Aldrich (Steinheim, Germany), while methanol and diethyl ether from Merck (Darmstadt, Germany). Since standard HCAL is not commercially available, and therefore pure HCAL was obtained from lactonization of HCA in the fruits of *G. atroviridis* using the reported procedure [26]. From CZE analysis of an HCAL solution at 1000 ppm, HCA and other carboxylic acids in the HCAL solution were not detected. Therefore, HCAL at least 96%, in comparison with total carboxylic acids, was obtained in this work, where the maximum amount of its impurities is estimated from limit of detections (LODs) of HCA and other carboxylic acids (13, 3.9, 0.9, 12 and 10 ppm for HCA, OA, MA, CA and TA, respectively). Herbal products of *G. atroviridis* were purchased from a market in Thailand, and their brand names cannot be disclosed.

2.2. Preparation of buffers

The phosphate buffers at pH 6.5–8.0 were prepared by diluting an appropriate amount of 500 mM Na_2HPO_4 and 5.0 mM TTAB, and then adjusting to the desired pH with H_3PO_4 to give the desired concentration of Na_2HPO_4 containing 0.5 mM TTAB. The borate buffer at pH 9.2 was prepared by weighing an appropriate amount of $Na_2B_4O_7{\cdot}10H_2O$, adding 5.0 mM TTAB and then dissolving this solution in water to give a pH 9.2 borate buffer containing 0.5 mM TTAB. The borate buffers at other pHs were prepared by adjusting a $Na_2B_4O_7$ solution with 0.1 M H_3BO_3 or 0.1 M NaOH. A mixture of phosphate and borate buffers was prepared by diluting an appropriate amount of 100 mM $Na_2B_4O_7$, 500 mM NaH_2PO_4 and 5.0 mM TTAB, and then adjusting to the desired pH with 0.1 M NaOH to give the desired concentrations of $Na_2B_4O_7$ and NaH_2PO_4 containing

0.5 mM TTAB. All solutions were prepared in Milli-Q water, filtered through 0.45 μm filters, and then sonicated for 3 min in a water bath prior to CE analysis.

2.3. Preparation of standard solutions

Stock solutions of 2000 ppm HCA, HCAL, OA, MA, CA and TA dissolved in water were separately prepared by weighing an appropriate amount of each standard and then dissolving this in water. Each working standard solution was prepared by pipetting the appropriate amounts of each stock solution. Each solution also contained 0.5 mM TTAB and 200 ppm ICA as internal standard for the quantitative analysis.

2.4. Preparation of sample

The powdered herbal product of *G. atroviridis* in 10 capsules was separately weighed, and then mixed together. The powdered product (1.25 g) was then extracted with 15 ml of water by vortexing for 5 min and sonicating for 30 min. The mixture was then centrifuged at 3000 rpm for 5 min. The solid residue was re-extracted. The first and second water extracts were combined. The appropriate amount of the water extract was then diluted with water to give a final sample solution containing 200 ppm ICA and 0.5 mM TTAB. Prior to CE analysis, the diluted sample solution was filtered through 0.45 μm filters, and then sonicated for 3 min in a water bath.

2.5. CE conditions

All CE experiments were performed on a 5010 Beckman CE Instrument (Beckman Coulter, Inc., CA). An uncoated fused silica capillary used was 50 μm i.d. × 57 cm (50 cm to detector), and thermostated at 25 °C. The following conditions were used for CE analysis; voltage −20 kV, UV detection at 200 nm, and 0.5 psi pressure injection for 10 s. Prior to analysis each day, the capillary was rinsed with 0.1 M NaOH for 20 min followed by the running buffer for 20 min. The capillary containing the buffer was then conditioned by application of voltage at −20 kV for 30 min, in order to obtain stable reversed EOF by dynamic coating of TTA^+ at the capillary wall under the electric field. Between each injection, the capillary was rinsed with the running buffer for 3 min.

3. Results and discussion

3.1. Resolution optimization

The herbal products of *G. atroviridis* may contain minor organic acids, such as CA, TA and MA that may interfere with peaks of HCA and HCAL. Therefore, in initial work, CZE separation of HCA, HCAL and other carboxylic acids was carried out using a phosphate buffer at pH in a range of 6.5–8.0. TTAB was added at a level of 0.5 mM in the phosphate buffer to reverse EOF [21,23]. The reversed EOF and reversed polarity of applied voltage were chosen for fast migration of negatively charged carboxylate ions toward the detector. It should be noted that UV absorption of Br^-, as a counter-ion of TTA^+, can interfere with the detection of carboxylic acids at a wavelength of 200 nm. However, this effect may be less for major components of HCA and HCAL in the samples. For trace analysis of carboxylic acids, TTAOH, OH^- as a counter-ion of TTA^+, may be used, but it is more expensive than TTAB [22]. ICA was chosen as the internal standard for quantitative analysis because it is a tricarboxylic acid, and its structure is similar to HCA. In addition, ICA is not found in the sample. Fig. 2 shows electropherograms for separation of analytes using phosphate buffers at various pHs. It should be noted that, in this experiment, electrophoretic and electroosmotic mobilities have the same direction toward the detector, and therefore, the greater the migration time of the analytes in the same electropherogram, the smaller the electrophoretic mobility (μ) of the analytes. An increase in the buffer pH improved separation of the analytes, but baseline resolution of some analytes, $R_s > 1.5$, was not obtained. At buffer pH of 7.2, 7.5 or 8.0, the improved resolution was not obtained with increasing the concentration of phosphate buffers from 50 mM up to 100 mM Na_2HPO_4. The phosphate solution at pH > 8.0 was not used because the buffering capacity for $H_2PO_4^-$:HPO_4^{2-} is in a pH range of 7.2 ± 1.0.

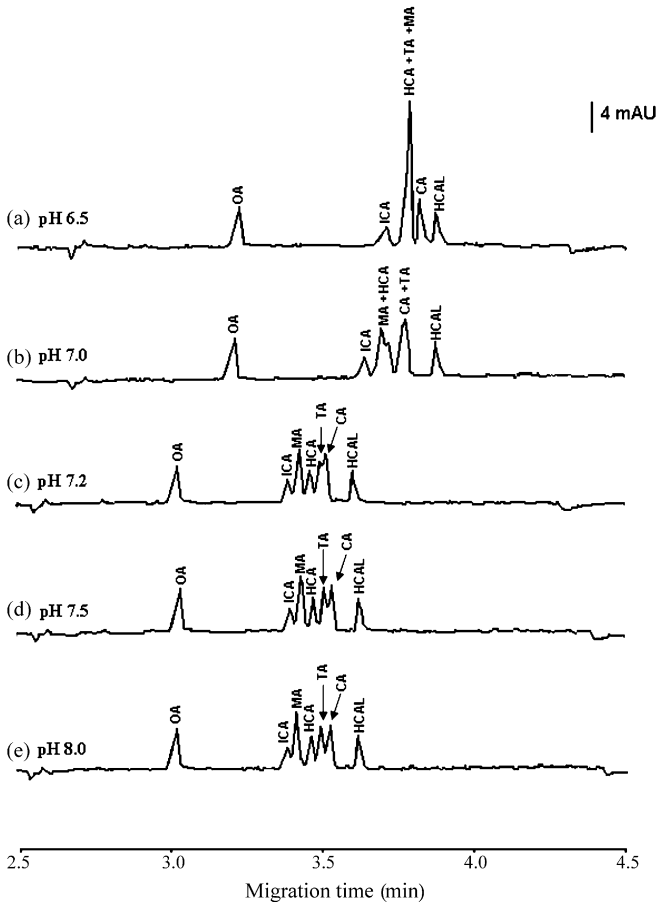

Fig. 2. CZE separation of HCA, HCAL and other carboxylic acid using phosphate buffers at various pHs. Buffer: 50 mM Na_2HPO_4, containing 0.5 mM TTAB, adjusted to pH with H_3PO_4. CE conditions: uncoated fused silica capillary 50 μm i.d. × 57 cm (50 cm to detector), temperature 25 °C, voltage −20 kV, 0.5 psi pressure injection for 10 s and UV detection at 200 nm.

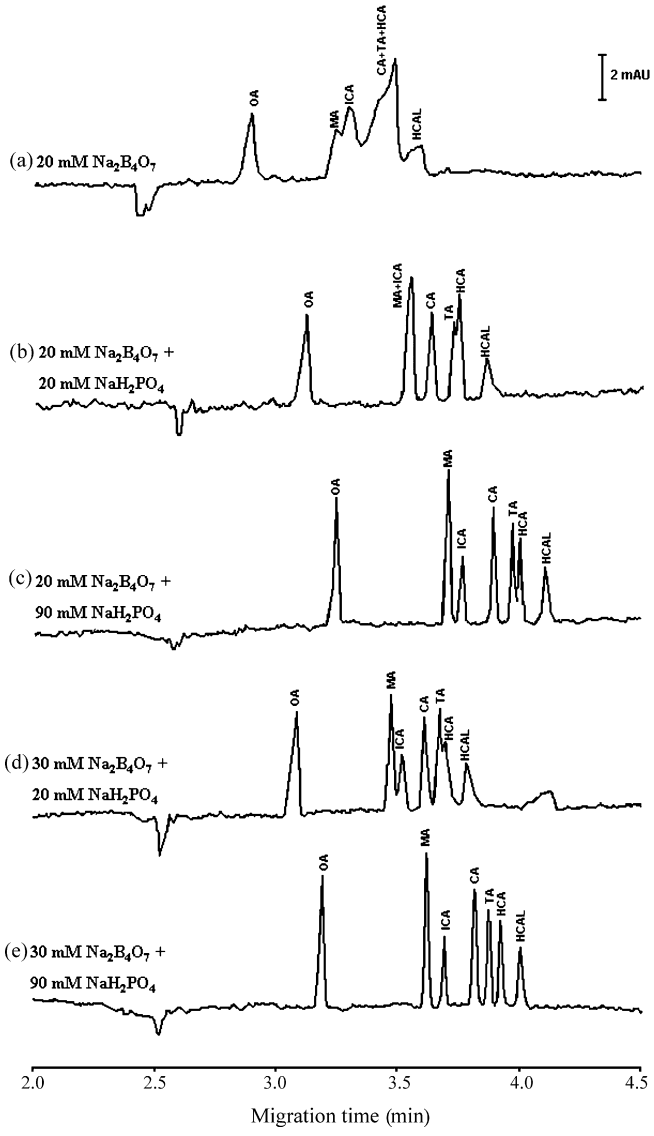

Fig. 3. CZE separation of HCA, HCAL and other carboxylic acids using (a) a pH 9.2 borate buffer and (b–e) a mixture of $Na_2B_4O_7$:NaH_2PO_4 containing 0.5 mM TTAB, adjusted to pH 9.2 with 0.1 M NaOH. Other CE conditions are shown in Fig. 2.

A pH 9.2 borate buffer, using 20 mM $Na_2B_4O_7$ containing 0.5 mM TTAB, was further used for separation of analytes, but baseline resolution was not obtained as shown in Fig. 3a. Either an increase in the concentration of $Na_2B_4O_7$ from 20 mM up to 65 mM or use of the borate buffer in a pH range of 8–10, did not give baseline resolution of all the analytes of interest. It can be seen from Figs. 2 and 3a that the migration order of analytes is different using phosphate and borate buffers, respectively. It should be noted that the phosphate solution at pH 9.2 and phosphate buffers at pH 6.5–7.2 also gave a similar migration order of the analytes. This indicates that buffer components affect the migration order, possibly due to the interaction between anionic analytes and boric acid in the borate buffer.

The mixed buffers of phosphate and borate at pH 9.2, using 20 or 30 mM $Na_2B_4O_7$, 20–90 mM NaH_2PO_4 and 0.5 mM TTAB, were used for separation of analytes. Addition of 20 mM NaH_2PO_4 in the borate buffer resulted in better separation as shown in Fig. 3b and d, and baseline resolution of all analytes was obtained using the pH 9.2 buffer containing 30 mM $Na_2B_4O_7$, 90 mM NaH_2PO_4 and 0.5 mM TTAB (Fig. 3e). Therefore, this buffer was chosen for separation and analysis of carboxylic acids of interest.

3.2. Validation of the method

LOD and limit of quantitation (LOQ) for analytes are defined as the analyte concentration giving signal-to-noise ratios of 3 and 10, respectively. Results of LOD and LOQ for HCA and HCAL are shown in Table 1. Without dilution of a 30-ml aqueous extract of 1.25 g of the powdered herbal product, sample detection limits (SDLs) of 0.031 and 0.038% (w/w) are obtained for HCA and HCAL, respectively, in the product, where SDL is equal to LOD adjusted to sample characteristics such as dilution, preconcentration and sample size. It should be noted that SDL is better or lower with the less amount of the extract volume, the larger sample weight, and the higher concentration factor. In this work, LODs and SDLs are sufficient for determination of major amounts of HCA and HCAL in our samples. Calibration plots were established by plotting the ratio of corrected peak area of the analyte ($A_{corr,ratio}$, y) to that of internal standard as a function of the analyte concentration (x) at six levels, where corrected peak area is defined as the peak area divided by the migration time (t_m). The concentration ranges and linear equations for calibration plots are listed in Table 1. Highly linear relationship between $A_{corr,ratio}$ and the concentration of each analyte was obtained with $r^2 > 0.998$.

The effect of sample matrix on accuracy of the method was investigated by spiking analytes with known amounts in the matrix such as water and the diluted solution of the sample. Each CE experiment was carried out for 10 runs. Results in Table 2 indicate high accuracy with the recoveries for spiked standard ranging from 99.8 to 101.1% with R.S.D. < 3%. In addition, the sample matrix was found to give no effect on the accuracy and precision due to the similar range of the recovery and R.S.D. for the standard spiked in the water and the sample.

The intraday and interday precisions in t_m and $A_{corr,ratio}$ were determined using the standard solution. For the intraday

Table 1
Calibration plots

Analyte	Concentration range (ppm)	Linear equation			LOD (ppm)	LOQ (ppm)
		Slope	Intercept	r^2		
HCA	50–300	0.0100	0.0147	0.9991	13	38
HCAL	50–500	0.0052	−0.0164	0.9985	16	47

Table 2
CZE analysis of the amounts of standard spiked in the matrix of the water (W) and the diluted solution of the sample (S)

Organic acid	Spiked (ppm)	Mean recovery (%) ±R.S.D. (%)	
		Matrix W	Matrix S
HCA	75	100.8 ± 1.1	97.8 ± 4.7
	180	100.1 ± 1.0	98.8 ± 4.2
HCAL	75	99.8 ± 2.1	99.7 ± 2.6
	250	101.1 ± 1.2	98.3 ± 2.9

$n = 10$ runs.

precision, the values of the average and R.S.D. were obtained from 10 separate runs each day, while 5 days for the interday precision. Results in Table 3 indicate high precision in t_m and $A_{corr,ratio}$ for the intraday, with R.S.D. < 1.0 and <4.0%, respectively. The acceptable values of R.S.D. for interday precision were found to be <4.1% for t_m and <4.6% for $A_{corr,ratio}$.

3.3. Application to real samples

In previous works [11–14], sample preparation for HPLC quantitative analysis of HCA and HCAL in genus *Garcinia* products was reported using an autoclave for extraction, typically at ~120 °C. However, it is well known that HCAL can be obtained from lactonization of HCA at high temperature [1,2]. Therefore, a comparison of sample extraction of samples was carried out at high temperature (~120 °C) using an autoclave and room temperature (~25 °C) using a simple procedure as mentioned in Section 2.4. Results show that an increase in temperature results in an increase in the determined amount of HCAL by 3–6% and a decrease in the determined amount of HCA 4 to 25%, while non-significant difference in the total determined amount of HCA and HCAL and the determined amount of CA. Therefore, simple extraction at room temperature was chosen for sample preparation without precipitation of pectinaceous material and any complicated procedure of sample preparation used in HPLC analysis [14,15].

Table 3
Intraday and interday precision of HCA and HCAL at 200 ppm

Precision	R.S.D. (%) and mean of t_m (min)		R.S.D. (%) and mean of $A_{corr,ratio}$	
	HCA	HCAL	HCA	HCAL
Intraday[a]				
Day 1	0.63 (4.07)	0.64 (4.17)	3.80 (1.49)	3.33 (1.26)
Day 2	0.82 (4.09)	0.84 (4.19)	3.03 (1.42)	3.26 (1.24)
Day 3	0.35 (3.94)	0.35 (4.03)	3.38 (1.40)	3.45 (1.24)
Day 4	0.64 (3.83)	0.66 (3.91)	2.70 (1.34)	3.73 (1.23)
Day 5	0.85 (3.72)	0.88 (3.80)	3.10 (1.34)	4.03 (1.28)
Interday[b]				
Overall	4.00 (3.93)	4.10 (4.02)	4.61 (1.40)	1.66 (1.25)

Values of mean in parentheses.
[a] $n = 10$ runs for each day.
[b] $n = 5$ days.

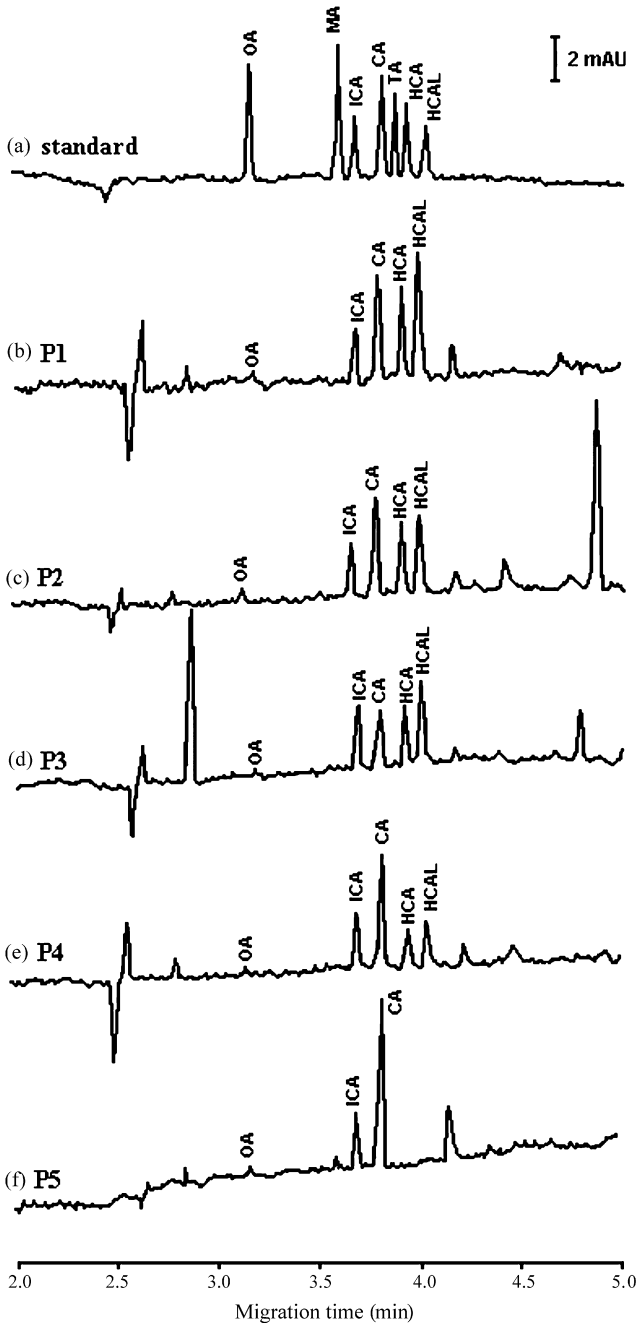

Fig. 4. An example of electropherograms of standard (a) and herbal products (b–e). Other CE conditions are shown in Fig. 2.

All samples, P1–P5, are claimed to contain *G. atroviridis* Griff extract. Fig. 4 shows an example of CZE electropherograms, and Table 4 shows the amounts of HCA and HCAL in the samples. The percentage, P (% w/w), of HCA, HCAL and other organic acids in each sample was determined from $A_{corr,ratio}$ and calibration plots as shown in Table 1. Since the samples have difference in the net weight per capsule, it is more useful to compare the weight of organic acids in a capsule of products. Organic acids in each sample were identified by spiking each standard. Samples P1–P4 were found to contain HCA, HCAL, CA and small amount of OA, while HCA

Table 4
Organic acids in herbal products determined by CZE (fourfold dilution of a 30-ml extract solution for 1.25 g of powdered products)

Sample	Net weight (mg/capsule)		Determined[a] amount			
	Labeled	Determined[c]	% w/w		mg/capsule[b]	
			HCA	HCAL	HCA	HCAL
P1	–	494.1 ± 20.8	1.23 ± 0.04	4.87 ± 0.07	6.08 ± 0.32	24.1 ± 1.1
P2	350	291.3 ± 13.5	1.36 ± 0.01	2.54 ± 0.06	3.96 ± 0.19	7.40 ± 0.38
P3	310	341.9 ± 20.4	1.01 ± 0.03	2.96 ± 0.02	3.45 ± 0.23	10.12 ± 0.61
P4	–	499.6 ± 19.0	0.57 ± 0.01	1.44 ± 0.03	2.84 ± 0.12	7.19 ± 0.31
P5	500	502.1 ± 15.5	nd	nd	nd	nd

nd = not detected.
[a] n = two batches and each batch for two runs.
[b] The S.D. values include S.D. from the determined net weight.
[c] n = the average from 10 capsules.

and HCAL were not found in sample P5 although the sample solution without dilution and the larger amount of the sample were used. This indicates that sample P5 is a fake product of *G. atroviridis* Griff. HCAL two to four times higher than HCA in samples P1–P4 may be obtained from lactonization of HCA at high temperature during the process of manufacture of herbal products such as drying, which is shown in our investigation. If the minor amounts of other carboxylic acids are wanted to determine, additional validation and different dilution factors should be made. However, this work is focused only on HCA and HCAL. In previous works on HPLC analysis, the amounts of HCA and HCAL were different depending on sources and species, such as 16–18% (w/w) HCA in the *G. cambogia* fruits [11], 10.3–12.7% (w/w) HCA and 0.8–1.8% (w/w) for HCAL in the *G. indica* rinds [13], 4.1–4.7% (w/w) HCA and 0.61–0.62% (w/w) HCAL in the *G. indica* leaves [13], 12.7% (w/w) HCA and 5.7% (w/w) HCAL in the *G. cowa* dried rinds of [14], and 1.7% (w/w) HCA and 0.8% (w/w) HCAL in the *G. indica* fresh leaves. From communication with the manufacturer, the herbal products of *G. atroviridis* contain other herbal plants for other purposes, and therefore, the HCA and HCAL contents in the herbal products should be less than those in the *G. atroviridis* fruits.

4. Conclusions

This work is the first report on CE for quantitative determination of HCA and HCAL. Baseline resolution of all analytes in standard and samples was obtained using a pH 9.2 phosphate–borate buffer of 30 mM $Na_2B_4O_7$ and 90 mM NaH_2PO_4, containing 0.5 mM TTAB, adjusted to pH with NaOH. Advantages of the developed CZE method over previous HPLC methods include faster analysis time within 5 min, better resolution of analytes and simpler sample preparation. In addition, high accuracy and precision were obtained from this CZE method. Thus, CZE can be used as an alternative method for determination of HCA and HCAL in herbal products of *G. atroviridis* Griff. In our future work, CZE will be used to compare HCA content in the *Garcinia* genus from various sources in Thailand, in order to obtain the best source of HCA used for herbal products.

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