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ORIGINAL PAPER

Capillary electrophoresis method for simultaneous analysis of caffeine, vanillin and ethyl vanillin in beverages

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Fast and simple capillary electrophoresis method was proposed for the simultaneous determination of caffeine, vanillin, and ethyl vanillin in beverages. The optimal separation conditions were 25 mM phosphate buffer at pH 8.5, containing 10% (v/v) acetonitrile and UV-absorption detection at the wavelength of 200 nm. Employing the optimum conditions, the three compounds were separated in less than 3 min. The procedure was validated with respect to linearity, limits of detection, limits of quantitation, (RSD%) accuracy (recovery), and precision (repeatability). Calibration curves with ($r^2 > 0.9986$) in the concentration range of 5–80 μg/mL were obtained for the three analytes. The limits of detection and quantitation in the range of 2.6-4.3 and 4.1-5.2 μg/mL were obtained, respectively. Recoveries of analytes in range from 92.1 to 104.4%, ($n = 3$) were attained. The validated method was successfully utilized for the analysis of the analytes in beverage samples.

KEYWORDS
beverages, caffeine, capillary electrophoresis, ethyl vanillin, vanillin

1 | INTRODUCTION

Caffeine (Figure 1A), chemically 1,3,5- trimethylxanthine, is a naturally occurring alkaloid and it is existed in different plant species and is present in their seeds, leaves, and fruits [1]. Caffeine is major components to the most type of soft drink, liquid dietary supplements and beverages [2–5]. Caffeine commonly is added to soft drinks as flavoring agent and is intentionally added to consumers attracted to these drinks. Vanillin, ethyl vanillin, and caffeine are often coexisting in many food and beverage.

Vanillin chemically it is 4-hydroxy-3-methoxybenzaldehyde is a phenolic aldehyde (Figure 1B). It is the principal constituent of the extract of the vanilla bean [6]. Ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde) (Figure 1C) is manmade ingredient, with more flavoring strength than vanillin and is utilized in formulation of simulation commodity [7]. Vanillin and ethyl vanillin are widely used flavoring ingredient in the food sector and have the greatest production in the world. Synthetic vanilla

Article Related Abbreviations: BGE, background electrolyte; EOF, electroosmotic flow; IS, internal standard; PTFE, polytetrafluoroethylene; RSD, relative standard deviation

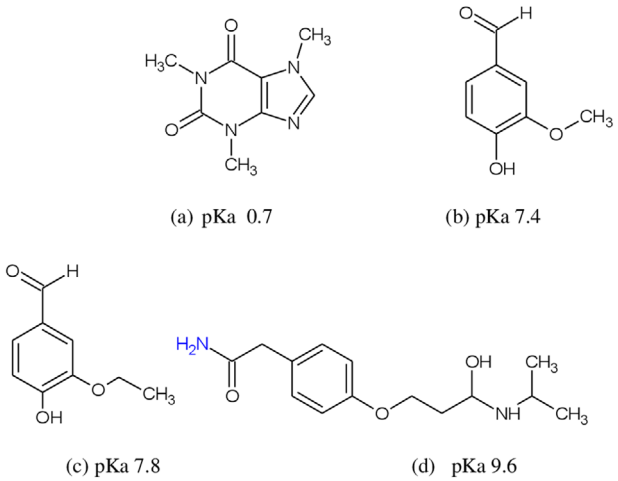

FIGURE 1 Chemical structures and pK_a values of (A) caffeine, (B) Vanillin, (C) Ethyl vanillin, and (D) Atenolol, the internal standard [36, 37]

flavorings, normally include vanillin and/or ethyl vanillin and other related compounds synthetically prepared from inexpensive raw materials, are commonly employed in the food sector in place of expensive authentic vanilla extracts.

Analytical methods for determining vanillin and ethyl vanillin from vanilla matrices such as vanilla extracts, juices, vanilla planifolia, milk powder were reported these include GC-MS [8,9], HPLC [10–12], and CE, mainly zone [13–15] or MEKC [16,17].

A number of analytical procedures have been utilized for the determination of caffeine in beverages, these techniques involve, spectroscopic methods [18–20], HPLC [4,6,21–23] CE [23–25], MEKC [26–28], laser diode thermal desorption mass spectrometry (LDTD-MS/MS) [29], ion mobility [30], and mass spectrometric detection techniques [31,32]. Electrochemical sensor for the simultaneous estimation of caffeine and vanillin in food and beverages were reported [33–35].

CE is an important separation technique with a high separation efficiency. Required a few minutes to equilibrate the CE system; after that, sample preparation is very simple since no more than a simple dilution of samples is generally required. The earlier described CE methods determined vanillin or ethyl vanillin, and caffeine individually or vanillin or ethyl vanillin simultaneously. To best of our knowledge, no CE method for the simultaneous measurement of vanillin, ethyl vanillin and caffeine in beverages was not reported. Therefore, the present study is devoted to validate a simple, and rapid, CE–UV procedure for simultaneously analysis of vanillin, ethyl vanillin, caffeine, in beverages.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Standard caffeine supplied from Lipomed, Cambridge MA, USA. Ethyl vanillin supplied from Toronto research chemicals (Toronto, Canada), vanillin supplied from Merck (Darmstadt, Germany) and atenolol supplied from MP Biomedicals, (Illkirch-Graffenstaden, France). Sodium hydroxide and acetonitrile were obtained from Merck (Darmstadt, Germany). Sodium phosphate dibasic heptahydrate was purchased from Sigma–Aldrich (Seelze, Germany). Methanol, hydrochloric acid (37%), ortho phosphoric acid (85 % were supplied from Sigma-Aldrich. Throughout the experimental work, double-distilled deionized water was used.

2.2 | Preparation of the electrolytes and standard solutions

Sodium phosphate dibasic heptahydrate buffer (25 mM) was prepared by dissolving 3.351 g with distilled water in 500-mL volumetric flask. The pH adjusted to desired pH with 0.2 M phosphoric acid. The solutions were filtered using 0.45 μm PTFE Millipore filter.

Three stock standard solutions of caffeine, vanillin, and ethyl vanillin each at concentration of 1000 μg/mL were prepared in the suitable way employing acetonitrile. Mixed working standard solutions were prepared by dilution with acetonitrile in an appropriate way at different concentrations ranged from 5.0 to 80 μg/mL.

2.3 | Sample and samples preparation

Different beverages namely, Pepsi, Coca-Cola, Red Bull, and Vanilla latte were obtained from local supermarkets. The preparation of the samples of Pepsi, Coca-Cola, Red Bull was composed of degassing in ultrasound for 2 min and filtering in a 0.22 μm Millipore filter (Millipore, USA), followed by injection in the CE instrument. Vanilla latte 1 g was dissolved in 25 mL boiling water then the volume completed to 50 mL with acetonitrile. Filtered and diluted five times before injection. All analysis was carried out in triplicate, and each replicate is means of three injections.

2.4 | Instrumentation

CE a P/ACE MDQ system from Beckman (Fullerton, CA, United States) was used. Separation was performed

using fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 39 cm and effective length of 28.5 cm with an internal diameter (id) of 77 μm, and outer diameter (od) of 375 μm. The new capillary was conditioned with 1.0 M sodium hydroxide for 10 min, 0.1 M sodium hydroxide for 3.0 min, water for 3.0 min and the BGE for 4.0 min. Prior to injection, the capillary was preconditioned using 0.1 M sodium hydroxide, water and the background electrolyte (BGE) for 2.0 min each. Samples were introduced using a pressure of 3.45 kPa for 10 s, and the analysis were executed under 25 kV voltage.

2.5 | Method validation

The validation of the procedure was evaluated in term of linearity, LODs, LOQs, repeatability, intra- and interday precision, and recovery. The linearity was assessed by analyzing at least six concentrations of the standards three times. Intra-day precision was examined by injecting standard solutions of caffeine, vanillin and ethyl vanillin at three concentration levels (10, 40 and 80 μg/mL) containing internal standard (IS) on the same day ($n = 6$). The repeatability interday precision was tested by analyzing standard mixtures of caffeine, vanillin and ethyl vanillin at concentration levels of (10, 40, and 80 μg/mL) containing IS for six consecutive days ($n = 36$).

The recovery study was determined by spiking 10, 30, and 60 μg/mL caffeine, and 10, 20, 60 μg/mL vanillin and ethyl vanillin and 25 μg/mL IS into 1.0 mL of Pepsi. The solution was made to 2 mL with buffer. For the Red Bull 10, 30, 60 μg/mL of caffeine, 10, 20, 60 μg/mL vanillin, and 25 μg/mL IS were spiked to 0.2 mL of Red Bull. The solution was made to 2 mL with buffer. The solutions were filtered and introduced into the CE.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of the separation conditions

To study the separation parameters by CE–UV, a solution consisted of the three analytes caffeine (10 μg/mL), ethyl vanillin (20 μg/mL), vanillin (20 μg/mL), and atenolol (10 μg/mL) were proposed. The benefit of using of organic solvent in the BGE are improving the solubility of less polar compounds and influence selectivity of separation. Therefore, solution of phosphate buffer in 10% (% v/v) acetonitrile as BGE was used.

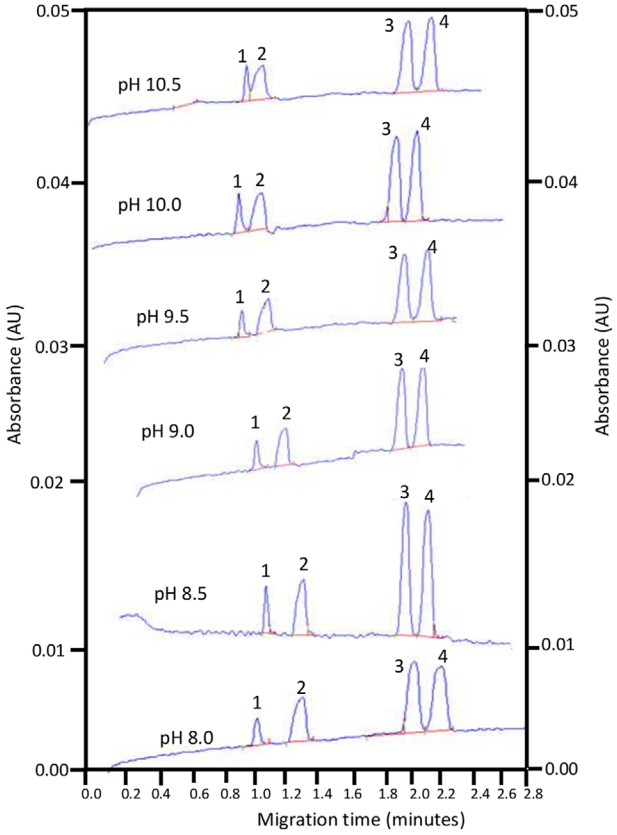

FIGURE 2 Effects of the pH of running buffer on the separation of caffeine, ethyl vanillin, and vanillin: CE conditions 25 mM phosphate buffer with the pH of the running buffer changed from 8.0 to 10.5; separation voltage 25 kV; injection sample 10 s under the pressure of 3.45 kPa; capillary temperature set at 25 °C

3.1.1 | Effect of the BGE pH

The critical factor to be studied in CZE is pH since it impacts the analytes charge and the silanol group ionization at the wall of the capillary, and thus affects the velocity of the electroosmotic flow (EOF). The pK_a values for vanillin, ethyl vanillin, caffeine, and atenolol are 7.4, 7.8, 0.7, and 9.6 [36, 37], respectively. Previous studies dealing with CZE separation of vanillin, ethyl vanillin [14,15] have investigated the use phosphate or borate buffer in basic pH range. In this work to maintain the caffeine, vanillin, ethyl vanillin, and the (IS) ionized; the pH value of the phosphate buffer (BGE) was examined within the range 8.0–10.5 at a 25 mM fixed buffer concentration. For the separation of caffeine, vanillin, ethyl vanillin, and the IS, the effect of the pH of the BGE was investigated in the pH range of 8.0 to 10.5 and the results are presented in Figure 2. The peaks were identified by spiking as follow peak 1, peak 2, peak 3, peak 4, for internal standard, caffeine, vanillin, and ethyl vanillin, respectively. The pH of the BGE was found

to be effectively impact the resolution of the three analytes. At pH ≥ 10.0, the resolution of the caffeine, vanillin, ethyl vanillin, and the IS was not good due to the high EOF and Joule heating.

3.1.2 | Effect of separation voltage

The effect of separation voltage on the migration time of the analytes was investigated in range of 15–30 kV. As expected, higher voltage resulted in shorter migration time. Twenty-five kiloVolt was selected as optimum separation voltage as it give better resolution with shorter analysis time. CZE methods have been reported using the same value of separation voltage [14, 15].

3.1.3 | Effect of capillary temperature

The effect of capillary temperature was also investigated in range of (20–30°C). As the temperature is increased, migration time decreases. 25°C was chosen as the working temperature for the electrophoretic analysis of the analytes. This optimum temperature is in agreement with reported methods in the literature [14-16, 23].

3.1.4 | Effect of sample injection time

Sample injection time was varied in range of 5—20 s at 3.45 kPa was in order to obtain a lower detection limit without affecting the quality of the peak shape and reproducibility, migration, and resolution. An injection time of 10 s offered best results and was selected for the rest of the studies.

Base line separation of the three compounds and the internal standard with the good peaks shape was obtained at pH 8.5. Thus, 25 mM phosphate buffer, pH 8.5, containing 10% (% v/v) of acetonitrile, applied voltage 25 kV, injection 3.45 kPa × 10 s, capillary temperature 25°C, were found to be optimum for the analysis of the caffeine, vanillin, ethyl vanillin, and the IS. A typical electropherogram of the three analytes and IS are presented in Figure 3. The peaks were identified by spiking.

3.2 | Validation of the analytical method

A number of reported studies have demonstrated that the use of internal standard is essential to achieve good repeatability in CE, so as to compensate for minor variation of the migration time and injection errors [38,39]. Atenolol, was used as an (IS) at concentration of 10 μg/mL.

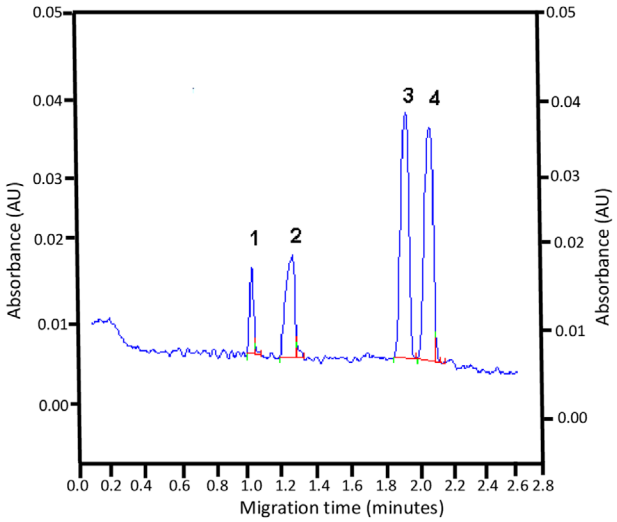

FIGURE 3 Typical electropherogram for a synthetic mixture; Caffeine (10 μg/mL), Ethyl Vanillin (20 μg/mL), Vanillin (20 μg/mL), and Atenolol (10 μg/mL). Obtained under electrophoretic conditions of 25.0 mmol/L phosphate at pH 8.5, fused-silica capillary column (50.2 cm length 77 mm id), hydrodynamic injection time of 10 s at pressure of 3.45 kPa, separation voltage of 25 kV and column temperature of 25°C. Peak identification 1: internal standard, 2: caffeine, 3: vanillin, 4: ethyl vanillin

The determination of caffeine, vanillin and ethyl vanillin was validated with respect to linearity, LODs and LOQs, inter- and intraday precision, and recovery [39].

Utilizing the optimum separation parameters, linearity was investigated at the concentration range of 5–80 μg/mL for each caffeine, vanillin, ethyl vanillin. The calibration curves were established by plotting the ratio of peak area (analyte/IS) (y) as a function of analyte concentration (x) in μg/mL. The LODs were calculated as the amount of the injected sample to yield a signal-to-noise ratio of 3, and the LOQs were taken as the amount of the injected sample to give an S/N ratio of 10. The LODs and LOQs of the method were measured to be 4.3, 3.1 and 2.6, 5.2, 4.5 and 4.1 μg/mL for caffeine, ethyl vanillin, and vanillin, respectively, which was higher than those reported by Ali et al. [33] using HPLC. The analysis time of the established CE is faster (~3 min compared to ~9 min in the HPLC. The results achieved are illustrated in Table 1.

Intra-day precision was examined by introducing standard mixtures of caffeine, vanillin and ethyl vanillin at three concentration levels (10, 40, and 80 μg/mL) containing IS on the same day ($n = 6$). In all situations, the relative standard deviation (RSD) for migration times and corrected peak area were less than 3.2 and 5.6 %, respectively, Table 2. The interday precision was tested by introducing standard mixtures of caffeine, vanillin, and ethyl vanillin at three concentration levels of (10, 40, and 80 μg/mL),

TABLE 1 Linearity and LODs, LOQs of the proposed method

Parameters	Caffeine	Ethyl vanillin	Vanillin
Linear Range (μg/mL)	5.0–80.0	5.0–80.0	5.0–80.0
Slope	0.1066	0.1226	0.1428
Equation*	$y = 0.1066x + 0.0162$	$y = 0.1226x - 0.0908$	$y = 0.1428x - 0.0231$
Correlation coefficient (r)	0.9986	0.9993	0.9996
LOD (μg/mL)	4.3	2.6	3.1
LOQ (μg/mL)	5.2	4.1	4.5

*Note: y = ratio of peak area (analyte/IS) x = analyte concentration.

TABLE 2 Intra- and interday reproducibility for the repeated injection of different concentrations of caffeine, ethyl vanillin, and vanillin

Concentration μg/mL	RSD% (migration time)			RSD% (corrected peak areas)		
Intra-day precision ($n = 6$)	Caffeine	Ethyl vanillin	Vanillin	Caffeine	Ethyl vanillin	Vanillin
10	1.3	2.9	3.1	4.3	4.4	2.9
40	1.3	2.4	2.5	3.5	3.3	2.5
80	0.9	3.0	3.2	4.8	5.3	5.6
Inter-day precision ($n = 36$)						
10	2.4	1.9	1.2	5.1	4.6	5.3
40	1.6	4.7	4.9	6.4	5.9	6.1
80	1.7	3.8	4.4	5.8	4.2	4.6

containing IS for six consecutive days ($n = 36$). Good precision as verified with the RSD for migration times and corrected peak areas of ≤ 4.9 and 6.4%, respectively, was obtained Table 2.

TABLE 3 Percentage recovery ($n = 3$) for determination of caffeine, vanillin, and ethyl vanillin in beverage samples

Type of samples	Sample content	Spiked STD	Found	Recovery %
Caffeine μg/mL				
Pepsi	55	10	62.80 ± 2.24	96.61 ± 2.6
		30	83.89 ± 1.35	98.69 ± 3.9
		60	116.20 ± 3.44	101.04 ± 1.9
Red Bull	32	10	40.27 ± 3.32	95.88 ± 3.48
		30	64.69 ± 1.67	104.34 ± 2.6
		60	94.16 ± 1.19	102.35 ± 1.6
Vanillin μg/mL				
Pepsi	0	10	09.46 ± 2.90	94.60 ± 2.23
		20	18.53 ± 0.29	92.65 ± 0.56
		60	60.16 ± 0.84	100.27 ± 1.10
Red Bull	0	10	09.21 ± 2.85	92.10 ± 2.37
		20	20.81 ± 1.65	104.05 ± 0.56
		60	60.31 ± 1.05	100.52 ± 1.43
Ethyl vanillin μg/mL				
Pepsi	0	10	10.44 ± 2.25	104.40 ± 1.31
		20	19.54 ± 0.991	97.70 ± 0.99
		60	59.54 ± 0.991	99.23 ± 1.82

The recovery study was evaluated by spiking Pepsi and Red Bull with caffeine standard at three concentrations level of 10, 30, and 60 μg/mL and ethyl vanillin and vanillin standards at concentration level of 10, 20, and 60 μg/mL. The recoveries percentage in the range 92.10 to 104.4%, were obtained Table 3. Typical electropherogram for spiked Pepsi is presented in Figure 4.

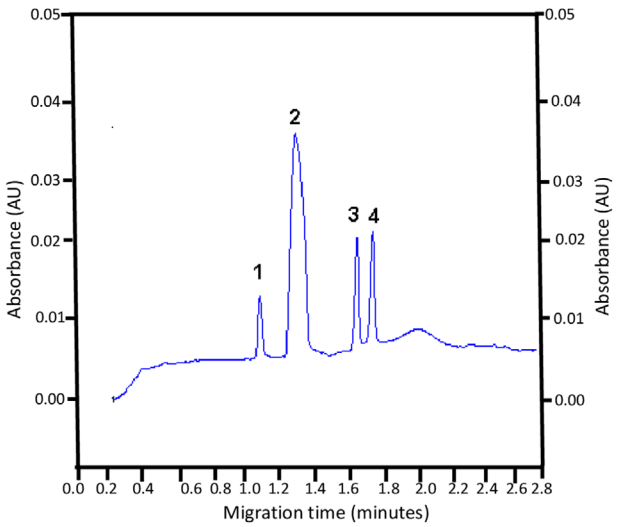

FIGURE 4 Typical electropherogram for Pepsi sample spiked with caffeine, ethyl vanillin, and vanillin, CE conditions and Peak identifications are as Figure 3

TABLE 4 Results of the analysis of caffeine, vanillin, and ethyl vanillin content in different beverage samples

Sample	Caffeine content µg/mL	Caffeine found ± SD µg/mL	Vanillin content µg/mL	Vanillin found ± SD µg/mL
Red Bull	32.0	34.08 ± 1.6	NL*	13.57 ± 0.5
Pepsi	11.0	10.9 ± 0.44	ND	ND
Cola-cola	9.5	9.9 ± 0.70	ND	ND
Vanilla latte	ND*	422.21± 0.61	NL*	303.23

ND: not detected NL: not label.

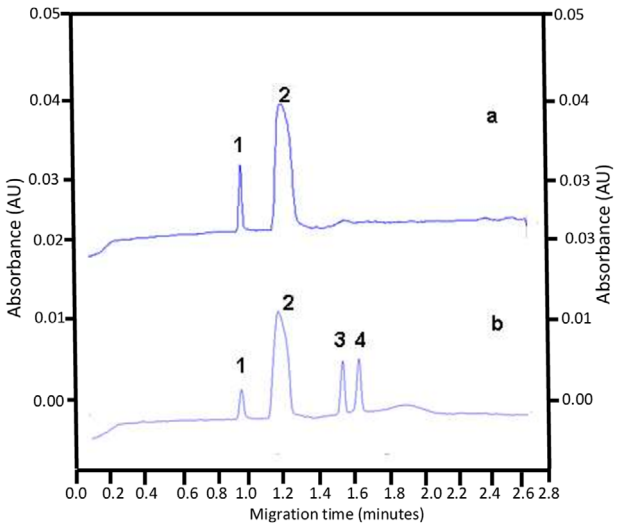

FIGURE 5 Electropherograms of real samples (A) Red Bull, (B) Pepsi, CE conditions, and Peak identifications are as Figure 3

3.3 | Analytical method application

The developed method was evaluated by determination of the three analytes in Red Bull, Pepsi, cola, and vanilla latte. The results are shown in Table 4. Typical electropherograms for analysis of Pepsi and Red Bull is presented in Figure 5.

4 | CONCLUDING REMARKS

Simple, fast, and inexpensive CE–UV method for simultaneous determination of caffeine, vanillin, and ethyl vanillin in beverages was validated. The procedure has good linearity, precision, and recoveries. This is the first CE procedure that able to simultaneous quantify caffeine and vanillin in energy drinks. Taking into consideration the results obtained in this investigation, the presented method can be an alternative, cheap and fast method in food industry.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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