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Article

Determination of parabens in sweeteners by capillary electrochromatography

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Parabens, common food preservatives, were analysed by capillary electrochromatography, using a commercial C18 silica (3 μm, 40 cm × 100 μm i. d.) capillary column as separation phase. In order to optimise the separation of these preservatives, the effects of mobile phase composition on the separation were evaluated, as well as the applied voltage and injection conditions. The retention behavior of these analytes was strongly influenced by the level of acetonitrile in the mobile phase. An optimal separation of the parabens was obtained within 18.5 minutes with a pH 8.0 mobile phase composed of 50:50 v/v *tris*(hydroxymethyl)aminomethane buffer and acetonitrile. The method was successfully applied to the quantitative analysis of paraben preservatives in sweetener samples with direct injection.

Uniterms: Capillary eletrochromatography/quantitative analysis. Parabenos/determination. Sweeteners/quantitative analysis.

Os parabenos, empregados como conservantes em alimentos, foram analisados por eletrocromatografia capilar, empregando uma coluna comercial recheada com partículas de sílica-C18 (3 μm, 40 cm × 100 μm d. i.) como fase estacionária de separação. Para otimizar a separação destes conservantes foram avaliados os efeitos da composição da fase móvel na separação, bem como a voltagem e as condições de injeção. O comportamento de retenção dos analitos foi fortemente influenciado pela proporção de acetonitrila na fase móvel. A separação dos parabenos foi alcançada em 18,5 min com uma fase móvel contendo tampão *tris*(hidroximetil)aminometano e acetonitrila na proporção 50:50 v/v. O método foi aplicado na análise quantitativa de parabenos em adoçantes empregando a injeção direta das amostras.

Unitermos: Eletrocromatografia capilar/análise quantitativa. Parabenos/determinação. Adoçantes/análise quantitativa.

INTRODUCTION

Capillary electrochromatography (CEC) is a separation technique that combines the selectivity of separations obtained by high-performance liquid chromatography (HPLC) with the miniaturisation and high efficiency obtained by capillary electrophoresis (CE), leading to a new technique with better properties. However, the electrophoretic and chromatographic interactions in CEC make it a more complicated analytical technique than either HPLC or capillary zone electrophoresis (CZE). The column used in CEC is a capillary, as is the separation column of CE. However, the column is packed either with a silica-based stationary phase or prepared to contain polymeric or silica-based monoliths, similar to those used in HPLC. Thus, the compounds migrate through the column according to their electric charge and according to their interaction with the stationary phase contained in the capillary column (Krull *et al.*, 2000).

The most frequent applications of CEC have been mainly as a variant of HPLC in which the flow of mobile phase through the column is maintained by an electric field rather than by applied pressure, resulting in an attractive analytical method that can be used to separate both neutral and charged compounds. In recent years, the number of publications related to this technique has been growing rapidly, probably because CEC has demonstrated some advantages, e.g., reduced time of analysis, higher resolution, enhanced peak efficiency and, mainly, the need for

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smaller amounts of solvents if compared to HPLC (Bragg *et al.*, 2008; Lerma-Garcia *et al.*, 2008; Musenga *et al.*, 2007; Valette *et al.*, 2005). In the pharmaceutical and food industries, rapid, inexpensive and efficient separation techniques are essential. Currently, most of these analyses are performed by HPLC, but the characteristics and availability of CE instrumentation have made CEC a technique with potential in these industries. (Lerma-Garcia *et al.*, 2008; Huo *et al.*, 2008)

The parabens are esters of *p*-hydroxybenzoic acid that differ in the alkyl group linked to the ester functional group. The parabens have antimicrobial functions and thus act as preservatives in food, pharmaceuticals and cosmetics (De Rossi, Desiderio, 2002). The antibacterial and antifungal activity of these compounds increases with increasing alkyl chain length, but this increase reduces the solubility of these compounds in water. Thus, the best preservatives are those that combine the features of being both soluble and antimicrobial. This property is achieved by mixing two types of parabens, the methyl and propylparabens, which are thus found with great frequency in foods, cosmetics and drugs (Panusia, Gagliardi, 2008).

There is a limit to the maximum amount of alkylparabens that can be introduced into foods, cosmetics and medicines, although possible side effects are not yet known. Hypersensitivity reactions have been reported with the use of hydroxybenzoates and it has been reported that parabens possess oestrogenic activity (Darbre *et al.,* 2002). According to the European Economic Community, the maximum content of parabens in cosmetics should be less than 0.4% w/w. However, 0.8% w/w has been found in the form of 4-hydroxybenzoic acid (which acts both as metabolite and as degradation product of parabens after hydrolysis under acidic or alkaline conditions) (Kokoletsi *et al.*, 2005). In pharmaceutical products, the limit is 1.0% w/w (Soni *et al.*, 2001, European Economic Community, 1993).

Studies on the separation of parabens have been developed using HPLC. Kokoletsi *et al.* (2005) separated the methyl and propylparabens found in raditidine oral liquid formulation using reversed-phase HPLC. They used a Nucleosil C18 column, UV detector at 254 nm and a mobile phase consisting of a mixture of aqueous ammonium acetate (0.5 mol L^{-1}), acetonitrile and methanol, with gradient elution. Samples of liquid pharmaceuticals were analysed and the chromatograms obtained presented a large number of peaks, indicating the presence of degradation product (4-hydroxybenzoic acid) and also the interaction between the parabens and the sorbitol existing in the sample.

Although CEC has been successfully used for the analysis of pharmaceutical compounds (Huo, Kok, 2008; Castro-Puyana *et al.*, 2010), few studies have been performed on the separation of parabens. The work of Huang *et al.* (2004) used monolithic columns based on esters of methacrylates as stationary phase. For all analytes it was observed that the capillary columns filled with more porous monoliths resulted in reduced retention times of the compounds and optimisation of the separation was obtained with columns containing fewer pores in the stationary phase. With this type of column, a good separation was achieved with a separation time of 7.0 min, for thiourea, sorbic acid, benzoic acid and ethyl, propyl and butyl parabens using a mobile phase composed of pH 3.0 phosphate buffer: acetonitrile, 35:65 v/v. In another work, Huang *et al.* (2006) used a monolithic capillary column based on polystyrene-divinylbenzene. This column was used due to its high stability against large pH variations of the medium. A third study, conducted by De Rossi and Desiderio (2002), emphasised the separation of parabens in cosmetics and drugs in the presence of its main metabolite, 4-hydroxybenzoic acid. In this study, they used a capillary column packed with C18 stationary phase. The optimum conditions of separation were obtained using a mobile phase consisting of 5.0 mmol l^{-1} ammonium formate buffer at pH 3.0: acetonitrile, 35:65 v/v.

The present study describes the separation of alkylparabens by capillary electrochromatography and their determination in sweeteners. A commercial capillary column filled with C18 silica particles was used. The conditions of separation were improved to achieve the best separation between the analytes, which were then quantified.

MATERIALS AND METHODS

CEC instrumentation

CEC experiments were performed with a HP CE instrument from Agilent Technologies (Waldbronn, Germany) equipped with a UV-visible diode-array detector and the option to apply pressures of 2-12 bar to the outlet and/or inlet vial. Data acquisition and processing were performed using HP Chemstation software.

A LC-10AD HPLC pump from Shimadzu (Kyoto, Japan) was used to condition the column.

Chemicals and sample preparation

HPLC grade acetonitrile (ACN) was obtained from J.T.Baker (USA), and potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). *Tris*(hydroxymethyl) aminomethane (TRIS) and thiourea were purchased from Sigma-Aldrich (Düsseldorf, Germany). Ethylbenzene,

propylbenzene, butylbenzene and pentylbenzene were the constituents of the test mixture from Fluka (USA). The solutions of the standard neutral mixtures were prepared by dissolving appropriate quantities of each compound in ACN to obtain a 10 mg mL^{-1} solution.

The standards of parabens, butylparaben, ethylparaben, methylparaben and propylparaben were obtained from Aldrich (USA). A standard mixture of parabens (10 mg mL^{-1}) in different volume ratios, as well as thiourea (5 mmol L^{-1}), were prepared. The analytical curves were constructed from three replicate measurements at each concentration in the range of 250 to 1500 µg mL^{-1} (5 points). Thiourea (5 mmol L^{-1}) was used as an electroosmotic flow (EOF) marker.

Commercial sweeteners (liquid samples) were obtained from a supermarket in Campinas, Brazil, and used as samples for the determination of parabens in this study. Without any sample preparation, the sweetener (200 µL) was fortified with 75 µL of standard analytes (10 mg mL^{-1}) and this mixture was completed with electrolyte until 1 mL. The solution was filtered and injected, and the optimum CEC conditions were applied.

CEC conditions

A C18-silica column (3 µm particles, 40 cm effective lenght × 100 µm inner diameter) was purchased from Agilent (Waldbronn, Germany). The column was placed in the CE instrument and was equilibrated with the mobile phase under the following stepping conditions: 5 kV, 10 kV, 15 kV, 20 kV, 25 kV and 30 kV, applying 5 bar of pressure for 15 min each. The last condition was maintained until a stable base line was obtained. Samples and standards were electrokinetically injected into the capillary for 5 s at a voltage of 10 kV. Separations were carried out using an electrical voltage of 30 kV, and 254 nm was selected as the detection wavelength. Mobile phases were prepared by mixing ACN and TRIS buffer (15, 25 and 50 mmol L^{-1}) in different volume ratios. 1 mol L^{-1} HCl was added to the mobile phase solutions until a pH of 8.0 was achieved.

RESULTS AND DISCUSSION

The first step was to test the performance of the capillary CEC column. The column was conditioned with ACN:H_2O 80:20 v/v, using the HPLC pump for 2 h. The performance was studied by analysing a neutral test mixture, thiourea, ethylbenzene, propylbenzene, butylbenzene and pentylbenzene. The electrolyte used for this study was ACN : 25 mmol L^{-1} TRIS, pH 8.0, 70:30 v/v. The separation was performed at 30 kV at a temperature of 25 °C. The samples were injected in the electrokinetic mode at 10 kV for 5 seconds. Table I shows the chromatographic parameters calculated for compounds of the test mixture and the relative standard deviation [RSD = (standard deviation/mean) × 100] calculated for three analyses of the mixture performed in one day. The maximum RSD values observed were 2.50, 1.88 and 2.68% for migration times, peak areas and peak heights, respectively. These data confirmed that the equipment and the column have good repeatability and can be used for the intended purpose.

The chemical structures of parabens used in this study are shown in Figure 1. As it can be observed from the chemical structures, parabens are neutral alkyl esters of 4-hydroxybenzoic acid. Due to the phenyl ring of the parabens, these compounds are UV-detectable at extremely low concentrations. Neutral solutes are separated in CEC by partitioning between mobile and stationary phase, as in HPLC, but they move through the column with the EOF, as in CE. The retention mechanism of parabens is determined by hydrophobic interactions between the C18 stationary phase and the nonpolar moiety of each analyte and by interactions between the polar mobile phase and sample molecules. In this study, the influence of operating parameters, such as the nature of the buffer electrolyte and its concentration, amount of organic modifier, applied voltage, and injection conditions were studied in order to optimise the CEC separation of these parabens.

Mobile phases consisting of TRIS, pH 8.0 (measured before mixing), modified with 50%, 70% and 80% of ACN, were used to study the influence of the modifier

TABLE I - Chromatographic parameters of the test mixture and relative standard deviation (RSD) of repeatability for three analyses of the mixture, performed in one day

Analytes	Migration time		Peak area		Peak height	
	Value / min	RSD / %	Value	RSD / %	Value	RSD / %
Ethylbenzene	10.27	2.5	180.00	0.93	26.10	2.7
Propylbenzene	12.33	2.5	251.33	0.63	29.63	2.3
Butylbenzene	15.17	2.4	220.97	1.22	20.90	1.4
Pentylbenzene	19.20	2.3	184.03	1.88	13.43	0.4

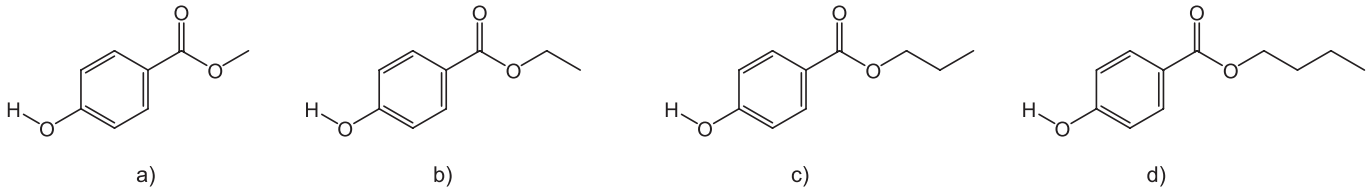

FIGURE 1 - Chemical structures of **a)** methyl, **b)** ethyl, **c)** propyl and **d)** butylparabens

on the separation of the parabens. The buffer TRIS was chosen because it has low mobility, which allows higher concentrations of buffer to be used without significantly increasing the current. The variation range of the current observed upon incorporating acetonitrile into the buffer solution within the concentration range 50-80% was 9 to 3.5 µA. A pH of 8.0 was chosen because the EOF at this pH has greater stability, when compared to lower pH values. The influence of the ionic strength of the mobile phase was evaluated using 15, 25 and 50 mmol L^{-1} TRIS. It was observed that both 50 mmol L^{-1} and 15 mmol L^{-1} of the buffer electrolyte affected the stabilisation of base line. Previous CEC studies (Yan *et al.*, 1994; Smith, Evans, 1994; Smith, Evans, 2005; Dittman *et al.*, 2005) have demonstrated that low buffer concentrations produce higher separation efficiencies, but in our experiment, the use of 25 mmol L^{-1} TRIS produced reproducible and highly efficient separations. An earlier paper (Huang *et al.*, 2006) had selected a mobile phase composed of ACN and 25 mmol L^{-1} phosphate buffer, pH 8.0, in the volume ratio of 50:50 v/v as the optimal condition for paraben analyses. Thus, some experiments were performed using phosphate buffer and acetonitrile as mobile phase, but under these conditions analyses were difficult, due to the long time required to achieve good stability of the base line and due to the instability of the current.

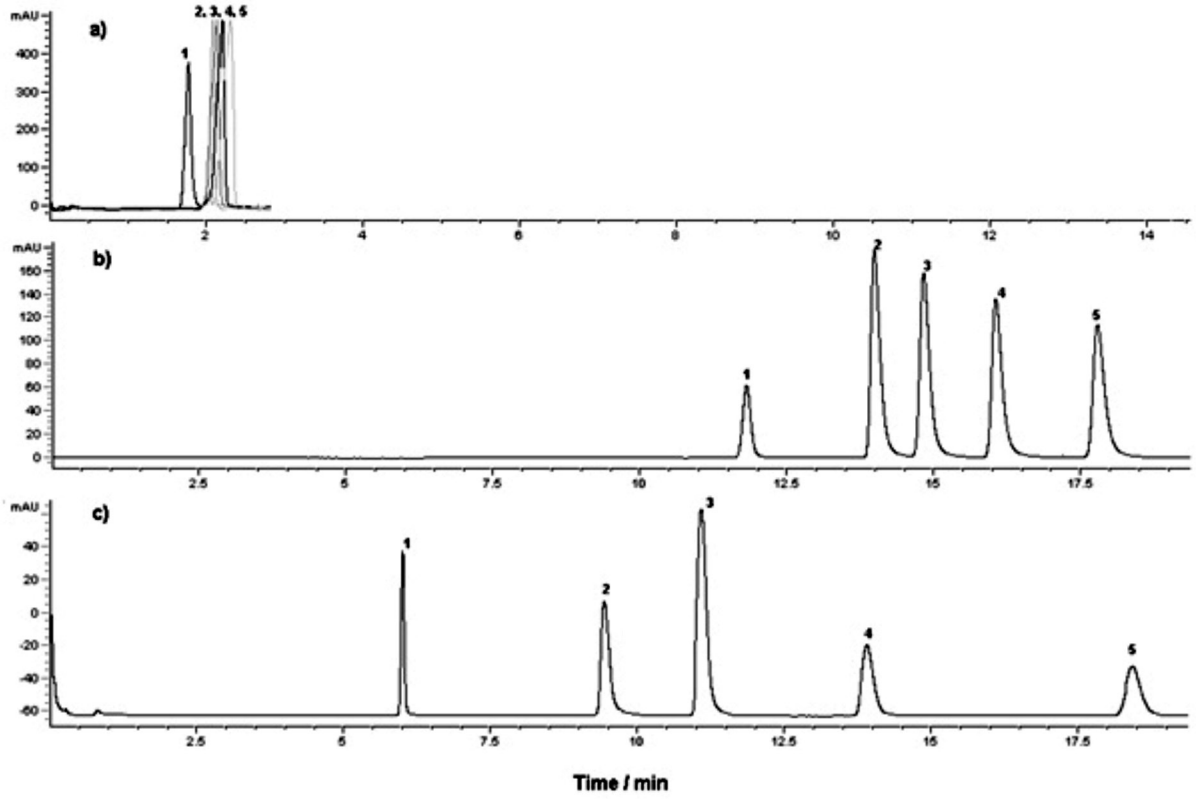

FIGURE 2 - Effect of mobile phase composition on the CEC separation of 5 mmol L^{-1}. **1)** thiourea and 1500 µg mL^{-1} **2)** methyl, **3)** ethyl, **4)** propyl and **5)** butyl parabens. Electrolyte: **a)** 25 mmol L^{-1} TRIS (pH 8.0):ACN, 20:80 v/v (each compound was injected separately) **b)** 25 mmol L^{-1} TRIS (pH 8.0):ACN, 30:70 v/v and **c)** 25 mmol L^{-1} TRIS (pH 8.0) /ACN, 50:50 v/v. Conditions: 3 µm C18 stationary phase, 40 cm packing × 100 µm i.d.; applied voltage: 30 kV; electrokinetic injection, 5 s, 10 kV; temperature: 25.0 °C; UV detection: 254 nm.

Variation of the organic solvent in the buffer was also examined, since it is known that this variable affects the analysis time. Longer analysis times are expected as the proportion of organic solvent is decreased because of the increased *k* value. With 80% acetonitrile, all compounds coeluted. In the presence of 50% acetonitrile the duration of a run for parabens was only slightly longer (1.0 min) in comparison with 70% acetonitrile, but with significant effects on resolution. Figure 2 displays electrochromatograms for the separation of parabens on a C18 stationary phase using binary TRIS-ACN mixtures. The results are in accordance with the literature (Schwer, Kenndler, 1991), with authors having reported the variation of the electroosmotic velocity according to the content of the organic solvent in the electrolyte, thus indicating that a reduction of various types of organic solvent in the buffer led to an increase in electroosmotic velocity. In our experiment, using the CEC technique, the same results were obtained, except with 80% acetonitrile. In this case, the presence of two effects can be responsible for unexpected behavior of thiourea when 80% acetonitrile was used. One of these effects is the retention of analyte by the stationary phase and the control of this retention by varying solvent strength in the mobile phase, i.e., a separation mechanism based on reversed-phase chromatography. The second effect is an increase in electroosmotic velocity when the proportion of organic solvent in the buffer decreases. Our experiments showed that, in the presence of 80% acetonitrile, the first effect prevails, leading to lower migration time of thiourea, when compared to 70% acetonitrile.

The effect of the applied voltage on the CEC separation of parabens was investigated using a mobile phase of ACN: 25 mmol L^{-1} TRIS buffer at pH 8.0, 50:50 v/v. As expected, retention times decreased when a higher voltage was applied. Figure 3 demonstrates the electrochromatograms obtained when 15, 20, 25 and 30 kV were applied. At 30 kV, the analytes eluted faster, with good resolution and efficiency. Based on these results, 30 kV was applied for this separation.

The injection conditions were studied by varying the injection time and the voltage of the electrokinetic injection. Injection times lower than 5 seconds and 10 kV showed a decreased separation performance and loss of symmetry of the peaks. The optimum conditions for the separation of the parabens were ACN: 25 mmol L^{-1} TRIS buffer at pH 8.0, 50:50 v/v, 30 kV with the solutions

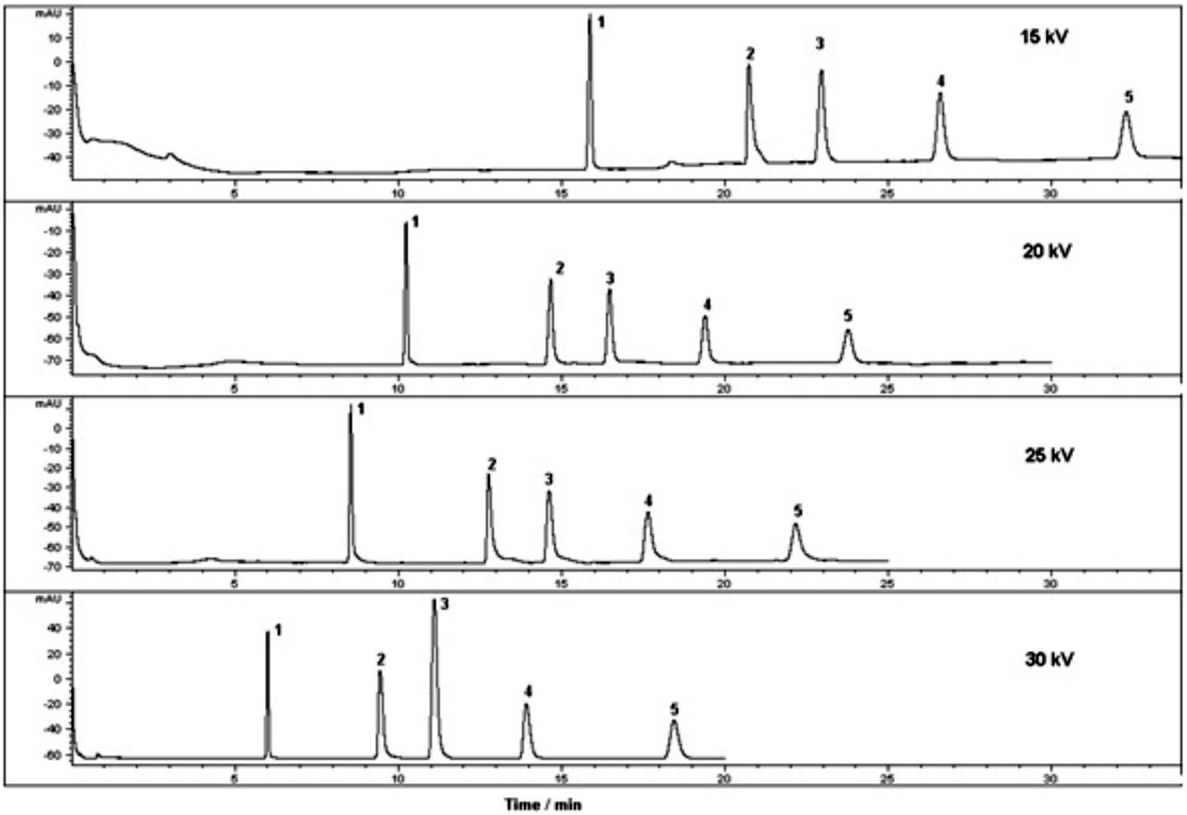

FIGURE 3 - Effect of applied voltage on the CEC separation of 5 mmol L^{-1} thiourea and 500 µg mL^{-1} parabens. Conditions: 25 mmol L^{-1} TRIS (pH 8.0):ACN, 50:50 v/v; 3 µm C18 stationary phase, 40 cm packing x 100 µm i.d.; applied voltage: 30 kV; electrokinetic injection, 5 s, 10 kV; temperature: 25.0 °C; UV detection: 254 nm.

TABLE II - Quantitative performance of parabens using the CEC method

Analytes	Relative regression equation (Y=A + BX)	R^2	Error		Calibration range / μg mL^{-1} (5 calibration levels)	Quantification limit / μg mL^{-1}
			A	B		
Methylparaben	Y = -48.43 + 0.89X	0.975	69.2	0.07	250 to 1500	19
Propylparaben	Y = -43.70 + 0.77X	0.955	82.0	0.08	250 to 1500	58

injected in the electromigration mode by applying 10 kV for 5 s.

The intra-day and inter-day injection repeatability was verified by analysing a methyl and propylparaben (1000 μg mL^{-1}) analyte mixture in three consecutive runs for three days, evaluating retention time, peak area and RSD% data. The maximum RSD values observed were 3.02 and 6.14%, for migration time and peak area, respectively. Under these experimental conditions, the CEC system enabled quantification of parabens in sweeteners. Table II reports the relative regression equations, the squared correlation coefficients and the quantification limits obtained. The concentration of methylparaben found in the sweetener was 2113 μg mL^{-1}, which corresponds to 0.21% w/v. For propylparaben, a concentration of 1199 μg mL^{-1} was observed, corresponding to 0.12% w/v. It was not possible to compare these values with the label information because the manufacturer did not declare these compounds on the labels. However, methyl and propylparabens are usually used in the range of 0.01 to 0.35% w/v of concentration in sweeteners.

CONCLUSIONS

In this study, a reliable CEC method was developed for the separation of parabens. The method can be applied for quantification of parabens in sweeteners that contain various concentrations of methyl and propylparabens. The results of repeatability and linearity also showed that, by using commercially available instrumentation and packed capillary columns, the CEC technique has the potential to be used routinely in food analysis.

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