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Determination of 2-Methylimidazole and 4-Methylimidazole in Caramel Colors by Capillary Electrophoresis

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ABSTRACT: The use of chemical preservative compounds is common in the food products industry. Caramel color is the most usual additive used in beverages, desserts, and breads worldwide. During its fabrication process, 2- and 4-methylimidazole (MeI), highly carcinogenic compounds, are generated. In these cases, the development of reliable analytical methods for the monitoring of undesirable compounds is necessary. The primary procedure for the analysis of 2- and 4-MeI is using LC- or GC-MS techniques. These procedures are time-consuming and require large amounts of organic solvents and several pretreatment steps. This prevents the routine use of this procedure. This paper describes a rapid, efficient, and simple method using capillary electrophoresis (CE) for the separation and determination of 2- and 4-MeI in caramel colors. The analyses were performed using a 75 μm i.d. uncoated fused-silica capillary with an effective length of 40 cm and a running electrolyte consisting of 160 mmol L^{-1} phosphate plus 30% acetonitrile. The pH was adjusted to 2.5 with triethylamine. The analytes were separated within 6 min at a voltage of 20 kV. Method validation revealed good repeatability of both migration time (<0.8% RSD) and peak area (<2% RSD). Analytical curves for 2- and 4-MeI were linear in the 0.4−40 mg L^{-1} concentration interval. Detection limits were 0.16 mg L^{-1} for 4-MeI and 0.22 mg L^{-1} for 2-MeI. The extraction recoveries were satisfactory. The developed method showed many advantages when compared to the previously used method.

KEYWORDS: *capillary electrophoresis, imidazole, caramel color*

INTRODUCTION

Alcoholic beverages and soft drinks, with few exceptions, contain caramel color at different levels of intensity. Although caramel color is not a flavor, many desserts and breads contain the substance, because its color is associated with good taste. As a result, this coloring is one of the most widely used additives in the food industry.

Caramel color is manufactured using a controlled heat treatment, in which food grade carbohydrates, such as glucose, sucrose, and invert sugar, are reacted in the presence of ammonium salts that promote color formation.[1] Caramel color is classified into four classes, depending on the reactants used in the manufacturing process. During caramelization using ammonia or ammonium salts as promoters (corresponding to class III and IV caramel colors, respectively), small quantities of substituted imidazoles, including 4-methylimidazole (4-MeI) and 2-methylimidazole (2-MeI) (Figure 1), are formed in side reactions.[2] The concentration of imidazoles in the caramel color product can vary widely, depending upon the manufacturing conditions, especially the composition of the catalyst and the type of carbohydrate used.[3] As a result of the use of caramel color, these chemical species have been identified in several foods and have also been detected in tobacco smoke.[4]

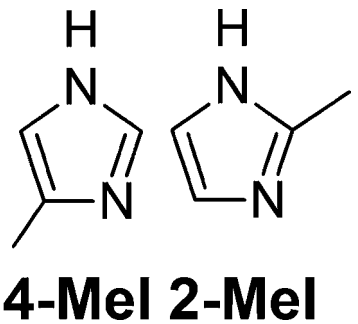

Figure 1. Isomeric structures of 4-MeI and 2-MeI.

The National Cancer Institute has identified 2- and 4-methylimidazoles as candidates for toxicity and carcinogenicity studies.[5] Imidazole derivatives can cause a state of hyperexcitation in animals. Additionally, these molecules can inhibit an enzyme (cytochrome P450) that is able to oxidize many known or suspected carcinogens.[1]

The federal regulatory agency responsible for the monitoring of food additives in Brazil (ANVISA) has established a concentration limit of 200 mg kg^{-1} for 4-MeI in caramel colors.[6] The content of 2-MeI is not regulated by either ANVISA or any other international agency, despite the potential toxicity of the compound.

Both gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used for the determination of imidazoles in various matrices.[7−12] The official protocol for caramel analysis is based on the extraction and purification of caramel using an organic solvent, sodium hydroxide, and Celite, followed by GC-MS analysis of 4-MeI in the form of the 1-acetyl derivative.[13] Practical limitations of these techniques

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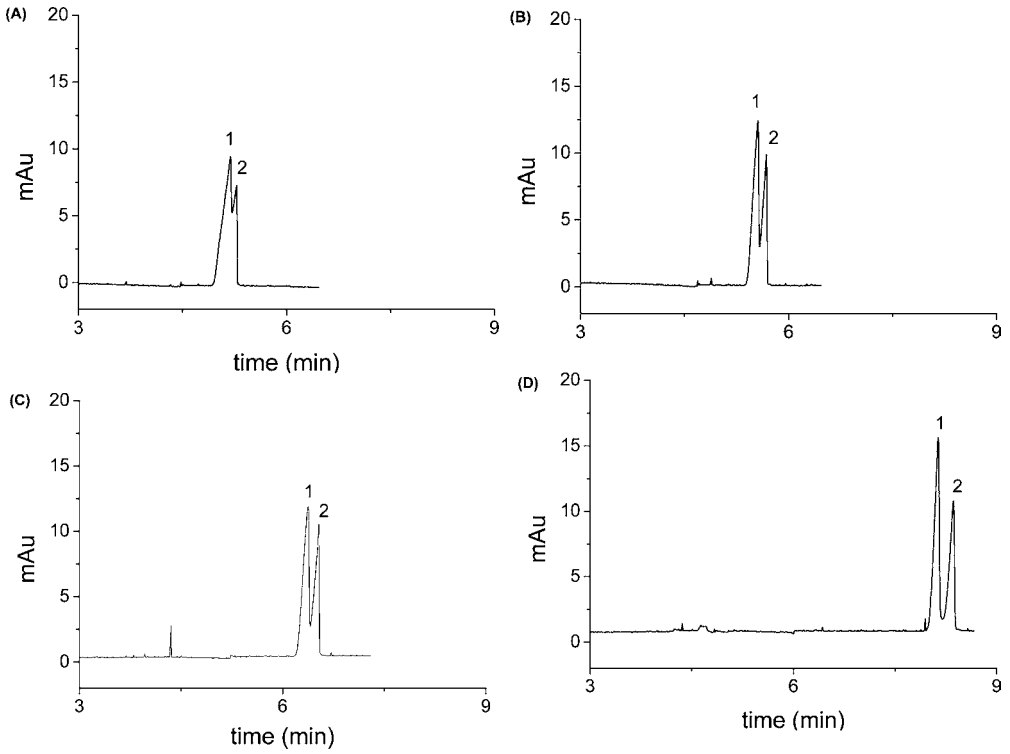

Figure 2. Optimization of the electrolyte concentration. Instrumental conditions: fused silica capillary, 75 μm i.d. × 360 μm o.d., 48.5 cm total length (40 cm to detector); hydrodynamic injection (12 s at 50 mbar); 20 kV separation voltage; T = 29 °C; direct UV at 227 nm; pH adjusted to 2.5 with TEA. Phosphate concentrations: (A) 20 mmol L^{-1}; (B) 40 mmol L^{-1}; (C) 80 mmol L^{-1}; (D) 160 mmol L^{-1}. Peaks: 1, 4-methylimidazole; 2, 2-methylimidazole.

include the need for expensive columns, high consumption of organic solvents, and laborious procedures for sample preparation. In this context, capillary electrophoresis (CE) is an attractive alternative technique for determination of imidazole species, because it offers high separation efficiency, low consumption of reagents, use of water-based electrolyte, and low total cost.

There have been only a few literature papers describing the separation and determination of imidazole derivatives using CE. Ong et al.[14] developed a method for the quantitative determination of four imidazole derivatives in pharmaceutical samples, using phosphate−borate buffer containing sodium dodecyl sulfate (SDS) as electrolyte. All four compounds were separated within 12 min. Kvasnička[15] proposed a method for the determination of 4-methylimidazole in caramel color, based on cationic separation of the sample by capillary isotachophoresis. No pretreatment of the sample was necessary, and the detection limit was found to be 5 ppm.

This work describes a novel free solution capillary electrophoresis (FSCE) methodology for the analysis of imidazoles in caramel color samples, enabling quantification of 2-MeI and 4-MeI in a single procedure. The method consists of dilution of caramel color in water and direct injection into a CE system.

MATERIALS AND METHODS

Chemicals. 2-MeI and 4-MeI were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid was obtained from QM (Minas Gerais, Brazil), triethylamine (TEA) was from Riedel-de Haen (Hanover, Germany), and methanol and acetonitrile were from Mallinckrodt (Pine Brook, NJ, USA). Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

Instrumentation. All experiments were conducted using a capillary electrophoresis system (Agilent Technologies, model HP 3D CE, Palo Alto, CA, USA) equipped with a diode array detector and a temperature control device to maintain at a constant temperature of 10 °C. Data acquisition and treatment employed HP ChemStation software (rev. A.06.01). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used (48.5 cm total length, 40 cm effective length, 75 μm i.d. × 375 μm o.d.). Samples were injected hydrodynamically, at 50 mbar for 12 s. The detection wavelength was set at 227 nm, and the applied voltage was 20 kV. The electrolyte was composed of 160 mmol L^{-1} phosphate and 30% acetonitrile, and TEA was used to adjust the pH to 2.5. On a daily basis, prior to the first analysis of the day, the capillary was conditioned by flushing with 1 mol L^{-1} NaOH solution for 5 min, followed by a 5 min flush with deionized water and a 15 min flush with electrolyte solution. The capillary was rinsed with fresh electrolyte solution for 3 min before each run.[16]

Sample Preparation. Class I, III, and IV caramel color samples were kindly donated by EPA Química Ltd.a. (Brazil) and Matrix Ingredientes (Brazil). An aliquot of 2.0 g of each color was diluted to 25 mL with deionized water in a volumetric flask. The solution was mixed and then filtered through a 0.45 μm pore size cellulose acetate filter disk. The filtrate was collected for CE analysis.

Preparation of Stock Solutions. Stock solutions of 2-MeI and 4-MeI (1 g L^{-1}) were prepared by dissolving appropriate amounts of the compounds in deionized water. These solutions were stored under refrigeration in brown bottles. Fresh working standard solutions were prepared daily by appropriate dilution of the stock solutions with deionized water.

Recovery Tests and Blind Samples. The accuracy of the method was assessed by performing recovery experiments. The imidazoles were added to samples at three different concentration levels. The recoveries were calculated by comparing the peak areas of the spiked and nonspiked samples with the peak areas of standard solutions at the same concentration, according to eq 1:[17]

$$\text{recovery}\,(\%) = \frac{A_S - A_{NS}}{A_{SS}} \times 100 \quad (1)$$

A_S = peak area of spiked sample, A_{NS} = peak area of nonspiked sample, and A_{SS} = peak area of standard solution.

To evaluate the accuracy of the results obtained by the proposed method, blind experiments were performed. In chemical analysis, blind experiments are used to test the validity of the measurement process.[18,19] Caramel color samples were spiked at concentrations in the range of 10−200 mg kg^{1-} to 2- and 4-methylimidazol by analyst 1; however, the identity of the sample as well as the concentration of the imidazoles within the sample was unknown to analyst 2, who performed the analysis of samples.[20]

Resolution. The resolution factor, Res, was calculated by the equation[21]

$$\text{Res} = \frac{2(x_{i2} - x_{i1})}{(w_1 + w_2)}$$

where x is the migration distance of analyte i, the subscript 2 denotes the slower moving component, and w is the width of the peak at the baseline.

RESULTS AND DISCUSSION

Method Development. Considering that the *N*-methylimidazoles are protonated at the imidazole ring (pK_a = 7.7), at pH below 8, phosphate buffer at low pH is the usual choice for the electrolyte system. The electrolyte system comprising phosphoric acid and triethylamine was chosen to start the optimization procedure. This electrolyte system has been described previously for determination of chiral separation of *N*-imidazole derivatives.[22−24] Under strongly acid conditions (pH 2.5), the imidazole derivatives are fully ionized, and there is suppression of the electroosmotic flow (EOF). As previously described by Sasse et al.[22] at low EOF the cationic analytes move only by electromigration, which leads to an increase in resolution and separation efficiency.

The effect of phosphoric acid concentration on separation was first studied in the range of 20−160 mmol L^{-1}, at constant pH (adjusted with triethylamine) and applied voltage. Resolution improved with increasing electrolyte concentration (Figure 2). However, higher concentration of electrolytes results in high conductivity, requires higher currents, and causes

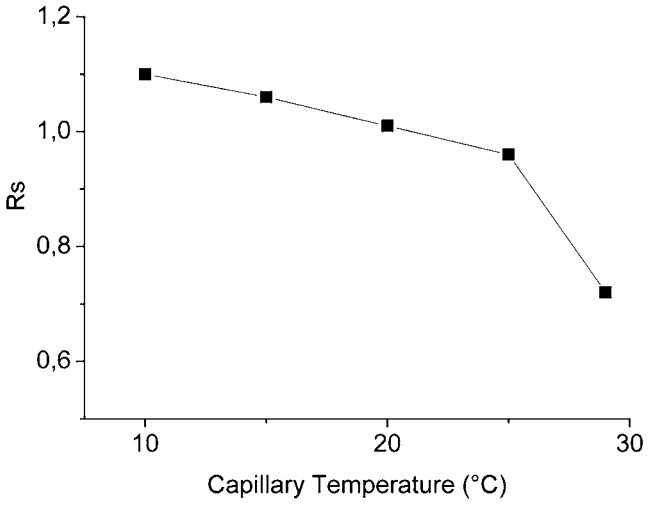

Figure 3. Effect of capillary temperature on peak resolution (Rs). The instrumental conditions were as described for Figure 2. The electrolyte used was 160 mmol L^{-1} phosphate, adjusted to pH 2.5 with TEA.

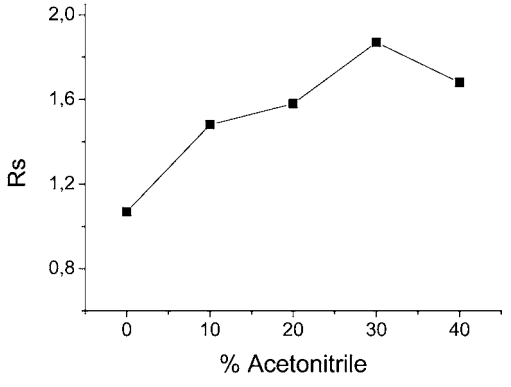

Figure 4. Effect of acetonitrile amount on peak resolution (Rs). Electrophoretic conditions were as for Figure 3.

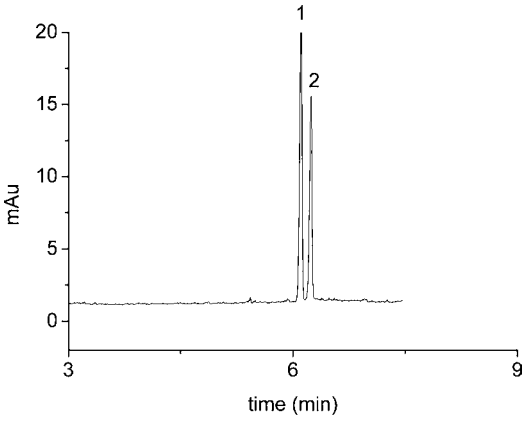

Figure 5. Electropherogram obtained under optimized conditions: fused silica capillary, 75 μm i.d. × 360 μm o.d., 48.5 cm total length (40 cm to detector); hydrodynamic injection (12 s at 50 mbar); 20 kV separation voltage; T = 10 °C; direct UV at 227 nm; pH adjusted to 2.5 with TEA. Electrolyte: 160 mmol L^{-1} phosphate and 30% acetonitrile. Peaks: 1, 4-methylimidazole; 2, 2-methylimidazole.

Table 1. Method Validation Considering Precision, Migration Time, and Peak Area Repeatability

analyte	migration time (min)	time, RSD[a] (%)	peak area, RSD[a] (%)
4-MeI	5.79	0.69	1.73
2-MeI	5.93	0.80	1.92

[a]RSD, relative standard deviation (10 consecutive injections).

Table 2. Method Validation in Terms of Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ)[a]

analyte	analytical curve	LOD[b] (mg L^{-1})	LOQ[c] (mg L^{-1})
4-MeI	Y = 1.274 + 3.335X	0.16	0.54
2-MeI	Y = 0.548 + 1.817X	0.22	0.65

[a]Five data points, with three replicate injections at each concentration level. [b]S/N = 3. [c]S/N = 10.

Table 3. Results of Recovery Studies Using Different Concentrations of 4-MeI and 2-MeI

concentration added (mg kg^{-1})	4-MeI	2-MeI
40.0	89.1 ± 2.0	92.8 ± 3.1
100.0	106.9 ± 1.8	107.4 ± 1.2
500.0	101.3 ± 1.9	103.8 ± 2.3

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Table 4. Nominal and Found Concentrations of Blind Samples of Caramel Colors

	nominal concentration		founded concentration		accuracy (%)	
blind sample	4-MeI	2-MeI	4-MeI	2-MeI	4-MeI	2-MeI
1	90.0	30.0	87.0 ± 1.80	30.2 ± 0.47	96.7	100.4
2	80.0	90.0	80.6 ± 0.88	88.8 ± 1.02	100.7	98.7
3	10.0	20.0	11.3 ± 1.32	22.9 ± 1.54	111.3	114.5
4	200.0	10.0	210.4 ± 4.18	9.6 ± 0.95	105.2	96.7
5	190.0		194.5 ± 1.51		102.4	

Table 5. Concentrations of Imidazole Derivatives in Commercial Caramel Color Samples

sample	4-MeI	2-MeI
caramel color class I	<LOD	<LOD
caramel color class III-1	163.1 ± 1.1	<LOD
caramel color class III-2	105.9 ± 1.0	<LOD
caramel color class III-3	99.40 ± 2.5	<LOD
caramel color class IV-1	146.8 ± 1.1	<LOD
caramel color class IV-2	163.3 ± 2.7	<LOD
caramel color class IV-3	100.8 ± 0.8	<LOD
caramel color class IV-4	114.4 ± 1.4	<LOD

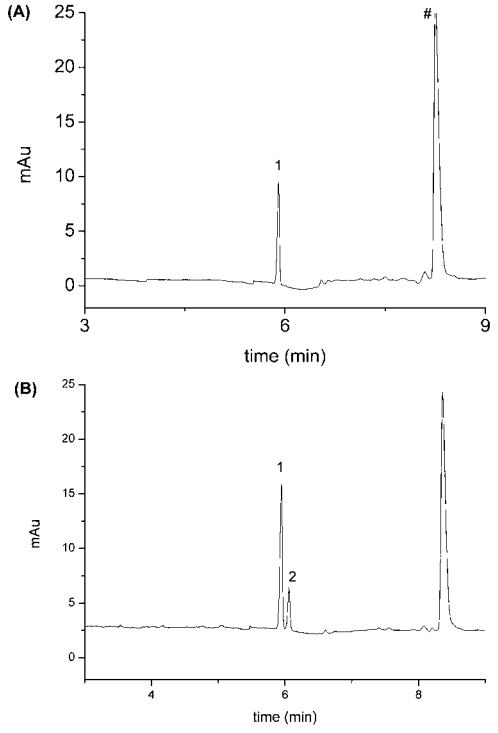

Figure 6. Electropherogram obtained for caramel color class IV (A) and spiked (B) samples. Electrophoretic conditions were as for Figure 5. Peaks: 1, 4-methylimidazole; 2, 2-methylimidazole; #, unidentified impurity.

greater joule heating than more diluted solutions.[25] The capillary temperature was therefore reduced to improve separation and resolve the problem of joule heating. The effect of capillary temperature on peak resolution is shown in Figure 3, where improved separation of 2-MeI and 4-MeI can be seen at lower temperature. A temperature of 10 °C was chosen as a compromise between a lower current and a higher resolution.

The complete resolution of the imidazoles required addition of a modifier to the electrolyte. Significant improvement in terms of selectivity, resolution, and separation efficiency can be achieved using organic solvents such as acetonitrile.[16] The influence of organic solvent on the separation was studied using acetonitrile at concentrations of up to 40%, with constant electrolyte concentration, pH, temperature, and applied voltage. The resolution improved with increasing acetonitrile concentration, up to 30%, and then declined (Figure 4).

The separation of 2-MeI and 4-MeI under the optimized conditions is shown in Figure 5. It can be seen that it was not necessary to add a chiral modifier to the electrolyte, despite the similarity in structure of the imidazole derivatives. This can be explained by the fact that the substitution patterns of the methyl groups in these molecules lead to differences in their electrophoretic mobility profiles. Imidazole is a five-membered heterocyclic aromatic compound with two nitrogen atoms. The nitrogen not bonded to hydrogen has a lone pair electron and is basic. Once imidazole is protonated, the two nitrogens become chemically equivalent in the aromatic ring. For 2-MeI, the alkyl group exerts an inductive effect by donation of electronic density through the bonds of the molecule. As a result, the positively charged nitrogen withdraws some electrons from the alkyl group bond in position 2, and the density of positive charge is lowered. The electron-donator effect of the methyl group attached at C-2 could therefore decrease the positively charged behavior of 2-MeI, compared to 4-MeI, after N-protonation. This enables separation of the isomers by FSCE.[26]

Method Validation. The figures of merit of the proposed method were determined following optimization of the experimental conditions. The precision was estimated using the relative peak area and relative migration time repeatability for 10 consecutive injections of a standard solution containing 10 mg L^{-1} of each analyte. Overall repeatability was better than 2% RSD (Table 1).

Analytical curves were constructed on the basis of peak area versus concentration. The analytical curves consisted of five points (n = 3 for each concentration level) between 0.4 and 40 mg L^{-1}. The analytical parameters are presented in Table 2. The technique showed good linearity over the evaluated concentration range (r > 0.998). The limits of detection were 0.16 and 0.22 mg L^{-1} for 4-MeI and 2-MeI, respectively.

The results obtained for the accuracy of the method are summarized in Table 3. The recoveries ranged from 89.1 to 107.4% for the concentration levels studied.

Analysis of Commercial Products. Three class I, III, and IV caramel color samples and the blind samples were analyzed using the proposed methodology. The results are summarized in Tables 4 and 5 (see also Figure 6). Compound identity was confirmed by spiking the extracts with standards. The content of 4-MeI in the samples was below the Brazilian legal limit (200 mg kg^{-1}), whereas 2-MeI was not detected. The accuracy of the blind samples ranged from 96.7 to 114.5% for the concentration levels.

A simple method for the analysis of 2- and 4-MeI in caramel color has been developed. The resolution, efficiency, and

selectivity of the technique were improved by decreasing the capillary temperature and by adding acetonitrile to the electrolyte system. Compared to other standard methods, the proposed CE method has the advantage that no solvent extraction step is required, because the caramel color is diluted in water. Satisfactory migration time repeatability and peak area repeatability were achieved, as well as good linearity and appropriate accuracy. The method was successfully applied to the analysis of commercial samples. The performance characteristics indicated that the proposed methodology should be suitable for the routine analysis of 2- and 4-MeI in caramel colors.

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Notes

The authors declare no competing financial interest.

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