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Quantification of 4-Methylimidazole in Class III and IV Caramel Colors: Validation of a New Method Based on Heart-Cutting Two-Dimensional Liquid Chromatography (LC-LC)

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ABSTRACT: 4-Methylimidazole (4MeI) is a nitrogen compound formed during the manufacture of class III and IV caramel colors. The European Commission has limited its content to 250 ppm. Two methods were compared to perform 4MeI quantification in caramels. The first one, currently used and considered to be the reference method, consists of a hot extraction of caramel color with dichloromethane and an analysis of the acetyl derivative of the extract by gas chromatography coupled to mass spectrometry (GC-MS). The second method is based on the heart-cutting two-dimensional liquid chromatography technique (LC-LC) to directly separate 4MeI from the other components present in caramel color sample (diluted in water) in <30 min. The accuracy profile validation method and the comparison between the results obtained with the two methods show that the new and completely automated LC-LC method is usable to quantify 4MeI in caramels.

KEYWORDS: 4-methylimidazole, caramel colors, two-dimensional liquid chromatography, gas chromatography−mass spectrometry, accuracy profile validation

INTRODUCTION

Many food products contain liquid caramel to improve their flavor and/or visual perception. Caramel colors are produced by heating carbohydrates (glucose, sucrose, invert sugar, etc.) in the presence of caramelization promoters: the obtained mixture is complex and responsible for the aromatic and colorant characters of caramels.

The use of ammonia or ammonium salt as promoters (corresponding to class III or IV caramel colors, respectively) involves the formation of undesirable neoformed compounds. Imidazole derivatives, such as 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole (THI) and 4-methylimidazole (4MeI), are formed from glucosylamine and fructosylamine, stemming from the reaction of ammonia on glucose, fructose, and sucrose.[1,2]

Toxicity studies of 4MeI showed a convulsive effect of this molecule, which can cause a state of hyperexcitation in animals such as mice and rabbits[3] or cattle.[4] This molecule can also inhibit an enzyme (cytochrome P450) that can oxidize many known or suspected carcinogens;[5] therefore, the European Commission has limited 4MeI concentration in class III and IV caramel colors to 250 ppm.

During the 1970s, few methods were developed to quantify 4MeI in caramel colors. They mainly used solvent extraction of pure caramel[6] or caramel mixed with Celite.[7,8] The extracting solvent is always a mixture of chloroform and ethanol (80:20), at basic pH to neutralize 4MeI. The analysis is performed by gas chromatography (GC), without derivation on a polar column containing an alkali-treated phase[7,8] or with acetyl derivation on an apolar column.[6] The official protocol for caramel analysis written by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is based on a method similar to that of Wilks et al.,[7] that is, extraction of caramel dried with Celite followed by GC analysis without derivation on a polar stationary phase. Thomsen and Willumsen[9] proposed a method based on ion-pair extraction of caramel with bis(2-ethylhexyl)phosphoric acid and analysis by ion-pair liquid chromatography with sodium dodecanesulfonate. More recently, the previous extraction followed by a gas chromatography−mass spectrometrty (GC-MS) quantification using isobutylchloroformate as derivatizing agent allowed a detection limit of 0.25 ppm to be achieved.[10] Two methods for the simultaneous determination of THI and 4MeI by liquid chromatography (LC) were also published: the first one[11,12] uses solid-phase extraction on a strong cation exchanger column followed by separation in reversed-phase mode (RPLC) on an apolar stationary phase with a basic mobile phase; in the second one,[13] the imidazole derivatives are extracted by supercritical carbon dioxide, and analysis is performed in hydrophilic interaction mode (HILIC) on a polar stationary phase column. The detection limit is 0.3 ppm with spectrophotometric detection at 215 nm.

All of these analytical methods require a long time for sample preparation. The objective of this paper is to present the validation of a new method for the determination of 4MeI in class III and IV caramel colors. This method is based on heart-cutting two-dimensional liquid chromatography (LC-LC):[14−16] the sample is fractionated on a first column, and the fraction containing the solute of interest is selected and injected online in a second column to complete the separation. Because of the high selectivity of the two-dimensional separation method, the sample

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preparation step can be reduced to a simple dilution of caramel in water and quantification of the solute of interest with a not very selective detector such as diode array UV absorbance detector is possible.[17] Moreover, this LC-LC method for 4MeI analysis in caramels can be completely automated; therefore, it is simpler and faster than the currently used methods, which are all based on offline extraction prior to chromatographic separation.

To demonstrate the specificity of the proposed LC-LC method for 4MeI, its results will be compared with those obtained with an orthogonal method used for a long time in Nigay S.A. and based on GC-MS. In the latter method, which is a combination of different published methods,[6,7] a semidry mixture of caramel and Celite is extracted with dichloromethane and, after the acetyl derivation of the extract, 4MeI is quantified by GC-MS. In a preliminary step, the GC-MS and LC-LC methods are validated using the accuracy profiles. According to this approach, developed by the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP),[18−21] accuracy (total error), which is a combination of trueness (systematic error or bias) and precision (random error), is measured under intermediate precision conditions to calculate an interval where a known proportion of future measurements will be located. By comparing this interval to an acceptance limit defined by the user, it is possible to simply decide whether the method is valid or not.

MATERIALS AND METHODS

Chemicals. HPLC-grade acetonitrile, methanol, and dichloromethane as well as acetic anhydride (96%), 25% ammonia solution, ammonium formate (97%), sodium hydroxide (99%), and Celite 545 were supplied by VWR (West Chester, PA). 4-Methylimidazole (purity = 98%) and 2-methylimidazole (purity = 99%) were purchased from Alfa Aesar (Ward Hill, MA) and Aldrich (Milwaukee, WI), respectively; 18.2 MΩ deionized water was filtered through an Elga PURELAB Option R 7 purification pack (Elga, Bucks, U.K.). Class III and IV caramel colors were obtained from Nigay S.A. (Feurs, France).

Equipment. GC experiments were performed on a HP 6890 chromatograph equipped with a HP 5972 mass detector (Hewlett-Packard, Walbronn, Germany). Ionization was carried out by electron impact. The capillary column used was a HP5MS (5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d.) with a 0.25 μm film thickness (Hewlett-Packard). Instrument control and data acquisition were performed using Chemstation version A.03.

LC experiments were performed on an Ultimate 3000 x2 dual liquid chromatograph (Dionex, Sunnyvale, CA), which includes two ternary low-pressure microgradient pumps, an autosampler, a thermal compartment with two 10-port two-position heart-cutting valves (equipped with a 1.5 mL loop), and a photodiode array detector. Instrument control, data acquisition, and compilation of results were made using Dionex Chromeleon software, V6.8.

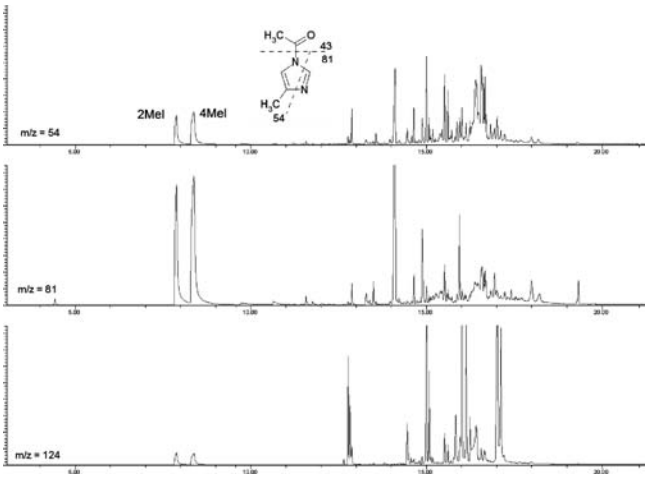

Figure 1. Analysis of a class III caramel color by the GC-MS method (SIM mode, *m*/*z* 54, 81, and 124). Peaks: 1, 1-acetyl-2-methylimidazole (internal standard); 2, 1-acetyl-4-methylimidazole. (Inset) Fragmentation scheme of 1-acetyl-4-methylimidazole.

Table 1. Elution Conditions Used in the LC-LC Method

		XBridge Shield RP18		
time (min)	flow rate (mL/min)	% ammonium formate 10 mM pH 11	% methanol	comments
0	0.7	100	0	collection of 4MeI between 5.8 and 7.3 min
10	0.7	100	0	
11	0.7	0	100	column washing
20	0.7	0	100	
21	0.7	100	0	column equilibrium
30	0.7	100	0	

		Hypercarb PGC			
time (min)	flow rate (mL/min)	% ammonium hydroxide % pH 11	% ACN	% methanol	comments
0	0.7	95	5	0	fraction recovery and 4MeI separation
20	0.7	95	5	0	
21	0.7	0	0	100	column washing
27	0.7	0	0	100	
28	0.7	95	5	0	column equilibrium
30	0.7	95	5	0	

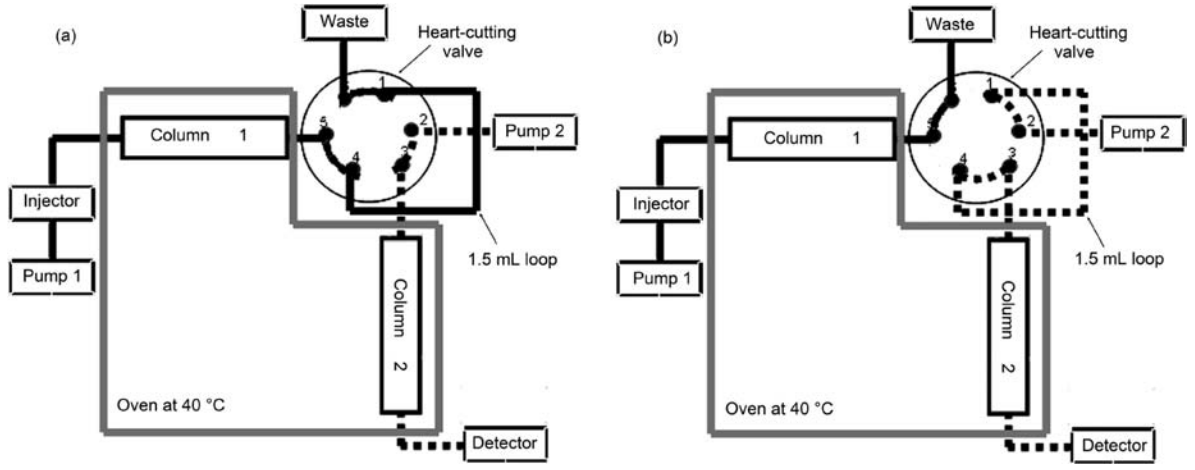

Figure 2. Experimental device for heart-cutting two-dimensional liquid chromatography: (a) first-dimension separation, heart-cutting of fraction containing 4MeI; (b) second-dimension separation, detection of 4MeI.

Liquid−solid extractions were performed on a B-811 system (Büchi, Flawil, Switzerland).

GC-MS Method (Reference Method). The currently used method for quantification of 4MeI in caramel colors at Nigay S.A. requires the weighing of 5 g of caramel. One hundred parts per million of an internal standard (2-methylimidazole (2MeI), which is not present in sample and can be separated from 4MeI by GC) is added to improve the analysis accuracy. The pK_a of 4MeI is equal to 7.7; the extraction of this molecule with an organic solvent required the addition of 3 mL of 3 N sodium hydroxide to basify the sample. Finally, 10 g of Celite 545 is added to dry the sample and to improve the extraction efficiency by increasing the specific surface of the sample.

The mixture is then extracted with hot dichloromethane[8] for 20 min with the liquid−solid extraction system. Dichloromethane is evaporated, and the dry extract is dissolved in 1 mL of acetic anhydride to form 1-acetyl derivatives of imidazoles.[6]

Two microliters of the obtained mixture is injected into the gas chromatograph at 240 °C with a split ratio of 30:1. The HP5MS column is heated at 75 °C for 10 min to separate the two 1-acetylimidazole compounds and then is cleaned by a rapid increase of temperature to 320 °C. For quantification of the two molecules, the mass spectrometer is used in the selected ion monitoring (SIM) mode at m/z 54, 81, and 124 (Figure 1).

LC-LC Method. In two-dimensional systems, the two dimensions must show a sufficient orthogonality so that the second dimension can separate the solutes which are not separated in the first dimension. The choice of the same LC mode (reversed-phase) in both dimensions, but with stationary phases of different selectivities (C18 silica in the first dimension and porous graphitic carbon in the second dimension), can allow the required orthogonality to be obtained without the technical problems associated with solvent nonmiscibility[22] and solute dilution.[17] The most retentive stationary phase is used in the second dimension to preconcentrate the solute recovered from the first column at the top of the second column.

Because of its polarity (log $P_{o/w}$ = 0.3) and its basic character (pK_a = 7.7), a correct elution of 4MeI (sufficient retention and slightly tailing peak) in RPLC requires working with a very low proportion of organic modifier in mobile phase and at alkaline pH, with columns stable in these conditions. The first- and second-dimension separations are performed on an XBridge Shield RP18 3.5 μm, 150 mm × 4.6 mm i.d. column (Waters, Milford, MA) and a Hypercarb PGC 5 μm, 100 mm × 4.6 mm i.d. column (Thermo, Waltham, MA), respectively. The elution flow rate in the two dimensions is 0.7 mL/min. The elution programs used in each dimension are presented in Table 1. Between two successive sample injections, the two columns are washed with 100% methanol. The XBridge Shield RP18 stationary phase of the first dimension, made of hybrid silica, is stable up to pH 12 and contains a carbamate group between the siloxane bridge and the alkyl graft, which allows the use of 10 mM ammonium formate aqueous solution basified with ammonium hydroxide at pH 11 as mobile phase. These minimum pH and ionic strength values are required to obtain a satisfactory peak shape for 4MeI. The Hypercarb PGC stationary phase of the second dimension, made of porous graphitic carbon, is stable up to pH 14, is recommended for the separation of polar compounds in RPLC[23,24] and is more retentive than XBridge Shield RP18 stationary phase. A mixture consisting of 5% of acetonitrile with 95% of water adjusted to pH 11 with ammonium hydroxide is required to correctly elute 4MeI on the PGC column. The solute of interest should be focused on the top of the second column when it is transferred from the first column with the 100% aqueous eluent.

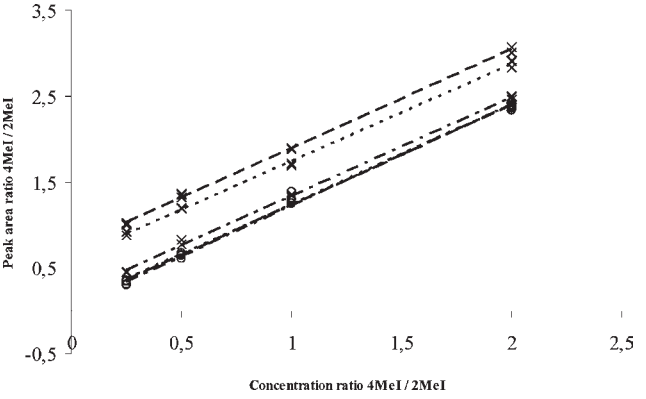

Figure 3. Graphical representation of the results obtained for the GC-MS method with calibration and validation standards: (○) calibration standards; (×) validation standards; (- - -) results of the first day; (— - —) results of the second day; (— — —) results of the third day.

The LC-LC system used is shown in Figure 2. The two columns are placed in the oven, the temperature of which is fixed at 40 °C. The detection wavelength is fixed at the maximum absorbance of 4MeI, that is, 217 nm.

The caramel sample is diluted 10 times in water before injection. The injected volume in the first dimension is 20 μL. The retention time of

Table 2. Quantitative Results of Validation of the GC-MS Method

		RSD			accuracy profiles	
concentration of 4MeI (ppm)	bias (%)	intraday repeatability (%)	interday repeatability (%)	precision (%)	lower limit (%)	upper limit (%)
25	−4.95	3.97	7.29	8.30	−34.55	24.66
50	4.37	3.38	1.10	3.55	−5.00	13.75
100	−1.12	0.77	2.22	2.35	−12.56	10.32
200	0.08	1.50	0.51	1.58	−4.32	4.48

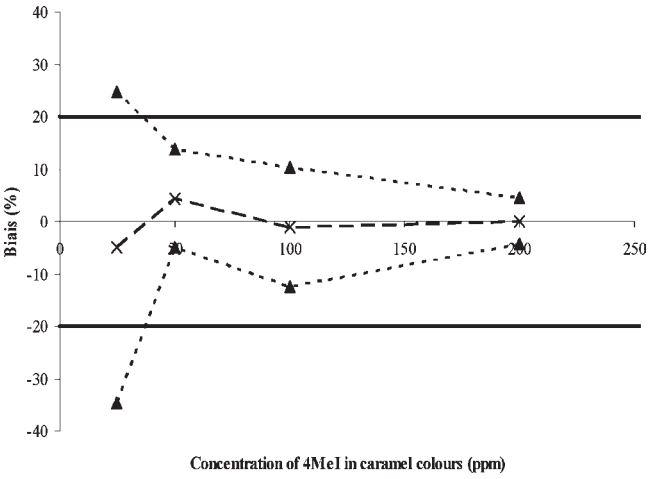

Figure 4. Accuracy profile representation for the GC-MS method: (— — —) bias; (- - -) accuracy; (—) acceptability limits (±20%).

4MeI in the first dimension being 6.3 min, the fraction eluted from the first dimension between 5.8 and 7.3 min is trapped in the loop (Figure 2a) before it is reinjected in the second dimension by switching the heart-cutting valve (Figure 2b). Therefore, the volume transferred between the two dimensions is 1 mL.

Method Validation with Accuracy Profiles. The objective of a quantitative analytical method is to be able to quantify as accurately as possible the solute of interest in its matrix. The difference between the result and the unknown true value of the sample (total error) must be lower than a predefined acceptance limit, which was fixed to 20%. The validation process is then to give guarantees that each of the future results that will be generated by the method will remain close enough to the true value. The proportion of measurements inside the acceptance limit must be larger than a given β-expectation tolerance interval fixed at 90%.

The V2 protocol of the SFSTP procedure[19] is as follows: the calibration standards are prepared by dissolving known concentrations of 4MeI in purified water, and the validation standards are obtained by spiking industrial caramel colors with known concentrations of 4MeI. Each calibration standard is analyzed twice, and the obtained results are used to draw the response function of the system. Each validation standard is analyzed three times for studying the intraday repeatability of the method. All of these analyses are repeated during three days with new calibration standards and new validation standards prepared from three caramels (a different caramel is used each day). The interday repeatability is calculated between days and caramels. From all results, bias and precision (which is the sum of intraday and interday repeatabilities) are calculated at each concentration, and the SFSTP protocol allows the calculation of the domain including 90% of the results for each concentration. Limits of this domain must remain within the acceptance limit (20%) to validate the quantification method. The concentration range selected for this validation is 10−500 ppm to verify

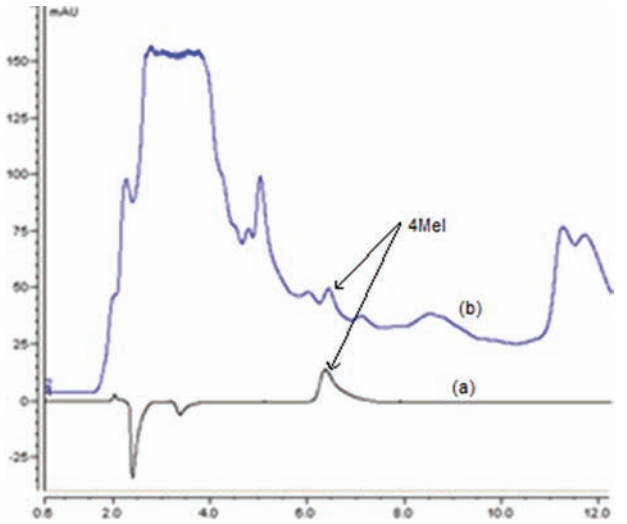

Figure 5. Chromatograms of (a) a 4MeI standard solution (5 ppm) and (b) a class III caramel color (diluted 10 times in water) on the XBridge Shield RP18 column with ammonium formate 10 mM, pH 11, as mobile phase. Injection volume = 20 μL. Detection wavelength = 217 nm.

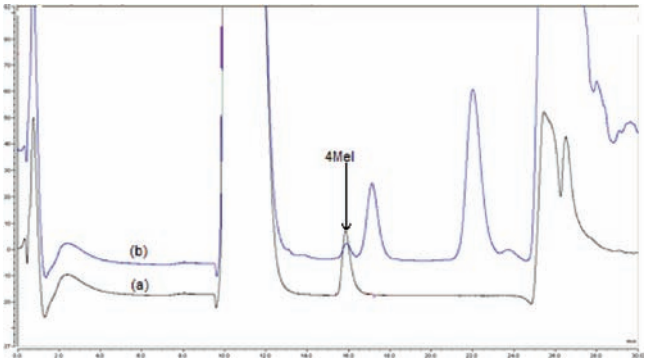

Figure 6. Chromatograms of (a) a 4MeI standard solution (5 ppm) and (b) a class III caramel color with the LC-LC method. Elution conditions: see Table 1.

the performances of the proposed LC-LC method for 4MeI contents placed on both sides of the European norm of 250 ppm. Application of the GC-MS method to class III and IV caramel colors produced by Nigay S.A. has shown that this limit is seldom exceeded.

RESULTS AND DISCUSSION

Validation of the GC-MS Method. Calibration and validation samples were prepared at four levels of 4MeI concentration: 25,

Table 3. Quantitative Results of Validation of the LC-LC Method

		RSD			accuracy profiles	
concentration of 4MeI (ppm)	bias (%)	intraday repeatability (%)	interday repeatability (%)	precision (%)	lower limit (%)	upper limit (%)
10	4.84	6.47	11.97	13.60	−43.67	53.34
20	−0.72	2.43	3.51	4.27	−15.88	14.43
50	−3.13	2.63	1.24	2.90	−11.24	4.99
100	−3.30	2.17	1.53	2.65	−11.37	4.77
200	−3.55	2.20	1.66	2.75	−11.94	4.84
500	−2.56	2.04	2.51	3.23	−13.99	8.87

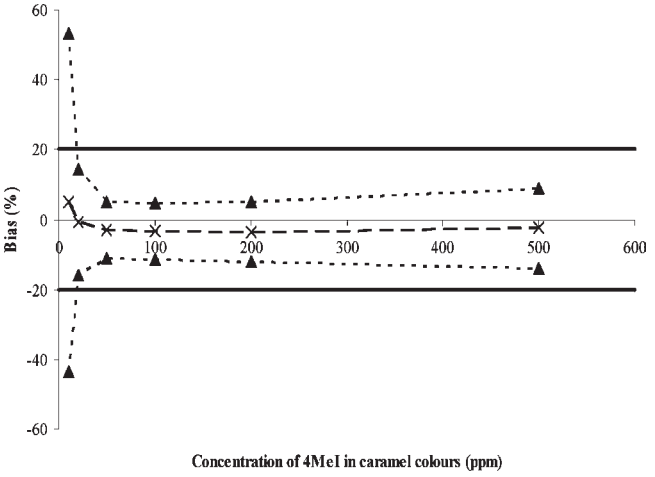

Figure 7. Accuracy profile representation for the LC-LC method: (— — —) bias; (- - -) accuracy; (—) acceptability limits (±20%)

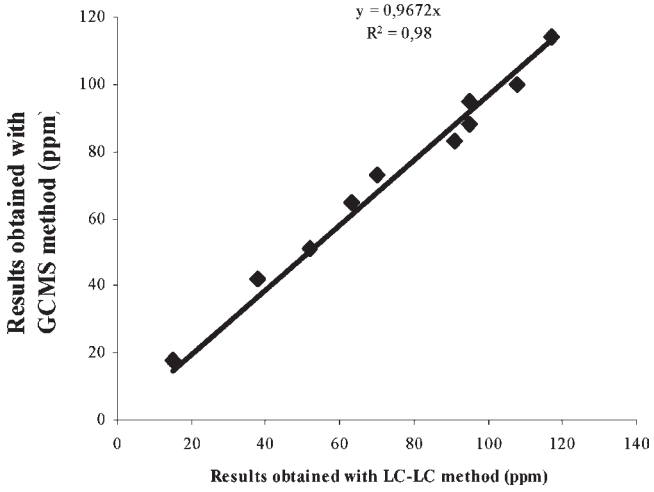

Figure 8. Comparison of 4MeI concentrations in industrial caramels determined with the GC-MS and LC-LC methods.

50, 100, and 200 ppm. The results (peak area ratio of 4MeI to 2MeI versus concentration ratio of 4MeI to 2MeI) obtained for calibration and validation standards are presented in Figure 3. On the one hand, the calibration curves of each day are superimposed. On the other hand, as validation standards are prepared by spiking each day a different commercial caramel color (which contains an unknown amount of 4MeI), the validation curves are not superimposed but are parallel to the calibration curves, which confirms the absence of matrix effects.

For each concentration of the validation range, bias, intraday repeatability, interday repeatability, and precision were determined with all of the results obtained with validation samples (Table 2). Bias is always lower than 5%, so the systematic error of the quantification method is low. Precision remains <4% (RSD%) between 50 and 200 ppm but becomes >8% (RSD%) at 25 ppm: the random error is important at the lowest studied concentration.

Bias and precision are used to calculate the lower and upper limits of the interval representing 90% of the results for each studied concentration (Table 2) by applying the SFSTP protocol.[20] The graphical representation of these limits allows the determination of the 4MeI concentration range where 90% of the results given by the GC-MS method are in the confidence interval of ±20% of the true value (Figure 4). These accuracy profiles show that, with these validation criteria (acceptance limit = 20% and expectation tolerance = 90%), the GC-MS method is no longer valid for concentrations of 4MeI in caramel of <40 ppm.

Validation of the LC-LC Method. Figure 5 shows the chromatograms obtained for a 4MeI solution and a caramel sample (diluted 10 times in water) under the conditions of the first dimension of the LC-LC method (XBridge Shield RP18 column and ammonium formate 10 mM, pH 11, as mobile phase). For these experiments, the absorbance detector was directly connected to the outlet of the first column. The peak of 4MeI is slightly asymmetric (As = 2.0) and, obviously, the separation power of this first dimension is not sufficient to analyze 4MeI in caramel color: there are numerous interfering compounds that coelute with 4MeI. A second separation step is required to completely isolate the 4MeI peak from the caramel matrix. This objective is reached with the LC-LC method, where the fraction containing 4MeI at the outlet of the XBridge Shield RP18 column (between 5.8 and 7.3 min) is selected in a loop and immediately injected in the Hypercarb PGC column. Indeed, from Figure 6, which shows the chromatograms obtained for the injection of a standard solution of 4MeI and the analysis of a caramel color with the LC-LC method, 4MeI seems to be completely separated from the other compounds present in caramel color. This new LC-LC method is rapid and automatic: an analysis takes 30 min, and it requires only a dilution of caramel in water and a filtration of the solution.

For the validation of the LC-LC method, calibration and validation standards were prepared at six levels of 4MeI concentration: 10, 20, 50, 100, 200, and 500 ppm. The curves of peak area versus concentration for 4MeI obtained with the calibration and validation solutions show no interference due to the caramel matrix: all of these curves are parallel (results not shown).

The new LC-LC method was evaluated in terms of trueness (bias), precision, and accuracy (Table 3). The bias observed with the LC-LC method (Table 3) is low (lower than 5% throughout the concentration range) and equivalent to those observed with the GC-MS method (Table 2). At low concentration of 4MeI, precision seems to be better for the LC-LC method (RSD% = 4.3% at 20 ppm) than for the GC-MS method (RSD% = 8.3% at 25 ppm). At 10 ppm (concentration not studied with the GC-MS method), the LC-LC method precision is decreased (RSD% = 14%) because this concentration is close to the quantification limit of 4MeI. This result compromises the use of the LC-LC method for concentrations of <20 ppm. The representation of the accuracy profiles (Figure 7) calculated according to the SFSTP protocol confirms this feature: the LC-LC method is able to quantify the 4MeI content in caramel samples (with 90% of the results included in the acceptability limit of ±20% from the true value) in the 20−500 ppm concentration range. For concentrations of <20 ppm, accuracy profiles move out of the confidence interval.

In conclusion, the advantages of the new LC-LC method are numerous. This method is validated by the accuracy profile protocol in a wider concentration range than the GC-MS method. In addition, it requires a very simple sample preparation, which consists only of a dilution of caramel color in water. The analysis protocol is performed on an automated instrument within 30 min, so the analysis rate is high.

Application to Industrial Caramels. Finally, to prove the specificity of the LC-LC method for the determination of 4MeI in caramel colors, the two orthogonal methods (LC-LC and GC-MS) were applied to the analysis of different industrial caramels. The results obtained are compared in Figure 8. A good correlation is observed between the 4MeI content quantified with the two methods. The GC-MS method is very selective thanks to the use of MS in SIM mode. Because the results obtained with the LC-LC method are just as good, it can be concluded that the latter method has a high level of selectivity. Finally, it can be noted that the 4MeI content of all analyzed caramel colors is between 20 and 120 ppm and, consequently, all of these caramels meet the European standard of 250 ppm.

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DEDICATION

This paper is dedicated to our colleague and great scientist, Dr. J. L. Rocca, who died September 18, 2009. We are grateful to Dr. Rocca for his invaluable advice and his great interest in the development of this work.

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