An Improved Method for the Determination of 4-Methylimidazole in Caramel Color

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An improved method for isolation and quantification of 4-methylimidazole (4-MeI) in caramel color is presented. The method consists of a methylene chloride extraction of a semidry mixture of the sample and Celite 545 (A registered trademark of Johns-Manville Celite Division, New York, N.Y.), followed by concentration and GLC analysis of the eluate. The GLC analysis is carried out using a base-modified column packing of 7.5% Carbowax 20M + 2% KOH. With this packing, the 4-MeI peak is symmetric, and the need for derivatization prior to analysis is avoided. Quantification is done using 2-methylimidazole (2-MeI) as an internal standard. Standard addition recovery experiments and reproducibility studies involving two analysts indicate that the method is acceptably accurate and has a precision of 2–3% relative standard deviation. The minimum level of 4-MeI detectable by this method is estimated to be 0.2 μg/g of caramel color.

Caramel color is manufactured by a controlled heat treatment, or "burning" process, in which food grade carbohydrates such as glucose, sucrose, or starch hydrolysates are reacted either alone or in the presence of catalysts which promote color formation. If ammonia or ammonium salts are employed as catalysts, the resultant product is termed ammonia process caramel. Additionally, ammonia process caramels may be manufactured with or without sulfur dioxide as a catalyst, depending upon the charge desired for the colloidal particles.

During the manufacture of ammonia process caramels, small quantities of substituted imidazoles, including 4-methylimidazole (4-MeI), are formed via side reactions which are incidental to those which result in color formation. However, the concentration of 4-MeI in the finished product can vary widely depending upon the manufacturing conditions and particularly upon the composition of the catalyst and the type of carbohydrate used (Allen, 1972).

At the 1969 meeting of the Joint FAO/WHO Expert Committee On Food Additives the question of the levels of 4-MeI in various types of caramel color was considered. As a result of these discussions, a concentration limit of 200 mg/kg for ammonia caramel colors of 20 000 EBC (European Brewing Council) units was recommended. The establishment of a concentration limit for 4-MeI in caramel color, together with the widespread use of caramel color in foods and beverages necessitates the availability of a method for determining 4-MeI which is accurate, precise, and sufficiently rapid for routine monitoring of caramel color samples.

In recent years, several methods for determining 4-MeI in caramel color have been published (Carnevale, 1975; Fuchs and Sundell, 1975; Komoto, 1973; Komoto et al., 1974; Wilks et al., 1973). However, extensive experience in our laboratories has shown that none of these methods is entirely satisfactory when accuracy, ease of use, and analysis time are considered. We have, therefore, found it necessary to modify our original method (Wilks et al., 1973), and these modifications are described in the present article.

In 1973, Wilks et al. (1973) described a method which utilized solvent extraction of the basic fraction, including 4-MeI, from the caramel color sample. After back-extraction of the basic compounds into dilute sulfuric acid, the quantitative determination of 4-MeI was carried out by direct GLC analysis of the neutralized aqueous extract. Standard addition experiments with model systems and with caramel color samples resulted in essentially 100% recoveries of concentrations of 4-MeI ranging from 25 to 200 μg/g of caramel color, and relative standard deviations for replicate analyses of less than 5%. A typical analysis required approximately 3 h to perform. However, the neutralized aqueous extracts were found to have limited stability to both light and temperature. In addition, repeated GLC analysis of the neutralized extracts resulted in build-up of Na_2SO_4 in the injection port and at the head of the GLC column and necessitated periodic down time for cleaning.

Komoto (1973) published a method which utilized strong anion exchange followed by weak cation exchange to isolate the basic fraction, which includes 4-MeI and other imidazoles, of caramel color samples. The imidazoles were eluted from the weak cation exchange resin with 1 N NaOH, and the neutralized eluate was then reacted with diazotized sulfanilic acid and analyzed colorimetrically. The total imidazole content of the eluate was reported as 4-MeI. Clearly, this method is not specific for determining 4-MeI in caramel color.

More recently, Komoto et al. (1974) have published a second article on this subject. In this work the ion-exchange technique employed for isolating the specific imidazole fraction was the same as that described earlier. However, a paper chromatographic separation of the individual imidazole, including 4-MeI, was described. Thus, the concentrations of the individual imidazoles, including 4-MeI, were determined. Standard addition experiments with caramel color samples resulted in acceptable recoveries of 4-MeI, although the lowest concentration employed was 156 μg/g of caramel color. Komoto et al. estimated the time required for a single analysis to be 13 h.

Carnevale (1975) has published a method which also utilized cation exchange for isolation of the basic fraction, including 4-MeI, from caramel color. The basic fraction was eluted from the cation-exchange resin with 4 N NH_4OH and extracted with chloroform. The chloroform was removed by evaporation, and the residue, dissolved in tetrahydrofuran, was allowed to react with acetic anhydride to form the *N*-acetyl derivatives. Imidazole was used as an internal standard and was added to the extract just prior to the derivatization step. Standard addition experiments with caramel color samples resulted in adequate recoveries as concentrations of 4-MeI greater than 125 μg/g of caramel color, although for lower concentrations (25 and 50 μg/g) the recoveries were less than

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90%. Carnevale indicated that the recovery of 4-MeI by the method appears to be concentration dependent. The time required for a single analysis was approximately 4 h.

Recoveries of 4-MeI from caramel color samples containing less than 125 μg of 4-MeI/g of caramel color are poor with both of these ion-exchange methods, a problem which we have also experienced in our laboratories (Kroplien, 1975). In addition, each of the methods is quite lengthy and, therefore, not entirely suitable for control purposes.

Fuchs and Sundell (1975) have published a method which consisted of a solvent extraction technique using separatory funnels, back-extraction of the basic compounds into dilute sulfuric acid, and reaction of the extracted imidazole with acetic anhydride to form the *N*-acetyl derivatives prior to GLC analysis. Quantitation was accomplished using 2-methylimidazole (2-MeI) as the internal standard. Recoveries of added known amounts of 4-MeI from caramel color samples were adequate over concentrations of 4-MeI ranging from 47 to 297 μg/g of caramel color. The time required for a single analysis was approximately 8 h.

While formation of the *N*-acetyl derivative of 4-MeI provides additional volatility to the compound and reduces peak tailing on polar GLC columns, e.g., Carbowax 20M, this step can be avoided simply by modifying the GLC column packing with a strong base such as KOH. When an internal standard is used, the point in the analysis at which the standard is added must be considered carefully. We have found through extensive experience that under the minimum conditions of solvent volume which enable 100% extraction of the 4-MeI from a sample of caramel color, only approximately 80% of the added 2-MeI is extracted. Thus, if 2-MeI is to be used as an internal standard, it should be added to the eluate rather than to the caramel color sample itself, unless the analyst is willing to considerably lengthen the analysis time by increasing the amount of extracting solvent.

It should be clear from the foregoing that none of the methods described above can be considered to be entirely suitable when the criteria of accuracy, precision, ease of acquisition, and analysis time required are considered. The method described below contains several improvements over our previous published method (Wilks et al., 1973), and we feel that it offers distinct advantages over the other methods as well. It is accurate, precise, and is rapid and facile enough to be used in a routine quality monitoring program.

EXPERIMENTAL SECTION

The following materials and reagents are required (the reagents should be ACS grade or equivalent where applicable. Materials: Pyrex glasswool, 22 × 300 mm chromatography column with PTFE stopcock (e.g., Kimax 17800), 150-mL polypropylene beaker (e.g., Nalge 1201), 250–mL round-bottom flask (e.g., Pyrex 4320), 75-mm powder funnel, 5-cm spatula, rotary vacuum evaporator, hot plate, pan for water bath, disposable Pasteur pipets, 5-mL volumetric flask. Reagents: acetone, Celite 545, methylene chloride, sodium hydroxide, and tetrahydrofuran.

After thoroughly mixing the caramel color sample by shaking or stirring, a 10.00-g aliquot is weighed into a 150-mL polypropylene beaker. Polypropylene is considered superior to glass because of its hydrophobic surface, which facilitates quantitative sample transfer. A 5.0-g portion of 3.0 N NaOH is added and thoroughly mixed to ensure that the pH of the entire sample exceeds 12. A 20-g portion of Celite 545 is added to the beaker, and the contents are mixed until a semidry mixture is obtained. This normally requires approximately 2 to 3 min. With samples of unusually high water content, e.g., double strength ammonia caramels, the resultant caramel color–Celite 545 mixture may be overly wet. In such cases, a 5.00-g aliquot of caramel color may be mixed with 2.5 g of 3.0 N NaOH and 15 g of Celite 545 and carried through the remainder of the analysis.

A plug of Pyrex glasswool is placed in the bottom of a 22 × 300 mm chromatographic column with PTFE stopcock. The caramel color–Celite 545 mixture is placed in the column with the aid of a 75-mm powder funnel. The column contents are settled by repeatedly allowing the column to fall vertically about 10 cm to a padded surface. When properly settled, the caramel color–Celite 545 mixture should occupy approximately the lower 150 mm of the column. Care should be exercised at this point to avoid a column bed which is either too loosely or too tightly packed. Loose packing will allow too rapid elution of the methylene chloride and possibly incomplete extraction. A too tightly packed column, e.g., the result of tamping down the column contents, can result in regions of the bed which are relatively inaccessible to the extraction solvent. This can also result in incomplete extraction. With the stopcock open, the column is filled with methylene chloride poured from the sample beaker. When the solvent reaches the glasswool plug, the stopcock is closed and the solvent is allowed to stand in contact with the bed for 5 min. The stopcock is then opened and the column is further eluted with methylene chloride until 200 mL have been collected in a 250-mL round-bottom flask. A 1.00-mL aliquot of 2-MeI internal standard solution (50.0 mg of 2-MeI/50.0 mL of methylene chloride) is added to the collected eluate. The 2-MeI is well separated from the 4-MeI under the GLC conditions employed and has not been found in caramel color of any type.

The bulk of the solvent is then removed from the eluate on a rotary vacuum evaporator operated at 45–50 kPa and with the round-bottom flask maintained at 35 °C in a water bath. The extract residue is transferred quantitatively to a 5-mL volumetric flask with a disposable Pasteur pipet, by rinsing the round-bottom flask several times with small (ca. 0.75 mL) portions of either tetrahydrofuran or acetone. Both solvents have been used with equal success. After mixing the contents thoroughly by several inversions of the flask, the extract is ready for GLC analysis. The extracts should be analyzed as soon as possible after their preparation, for stability problems have occasionally been encountered with extracts more than 1 day old.

The GLC analysis was carried out using a Hewlett-Packard Model 5711 gas chromatograph equipped with dual hydrogen flame detectors and a Hewlett-Packard Model 7671 autosampler. The column was glass, 1 m × 6 mm o.d. × 4 mm i.d., filled with 7.5% Carbowax 20M + 2% KOH on 90/100 mesh Anakrom ABS. The GLC parameters were as follows: carrier, nitrogen, 50 mL/min; hydrogen, 50 mL/min; oxygen, 80 mL/min; injection port, 200 °C; column isothermal, 180 °C; detector, 250 °C; sample size, 5 μL. All quantitation was done by the internal standard technique described by Ettre and Zlatkis (1967). Peak areas were determined by electronic integration using a Digital Equipment Corporation PDP 8/e computer system.

Figure 1 shows: (a) a chromatogram of a 5-μL injection of a standard solution containing 1 μg each of 2-MeI and 4-MeI; and (b) a chromatogram of a 5-μL injection of the extract of a caramel color sample containing 1 μg of added

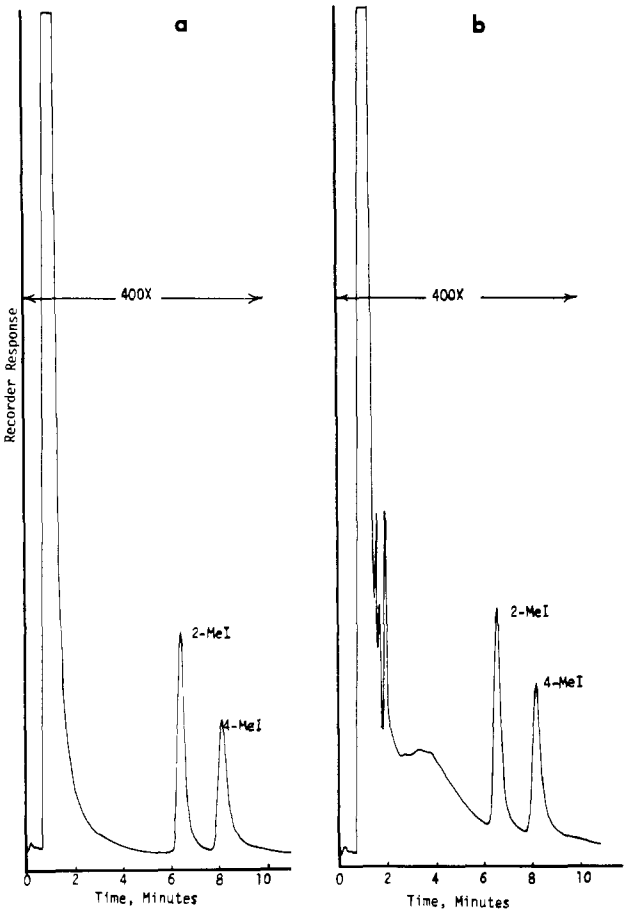

Figure 1. (a) A chromatogram of a 5-μL injection of a standard solution containing 1 μg each of 2-MeI and 4-MeI; and (b) a chromatogram of a 5-μL injection of the extract of a caramel color sample containing 1 μg of added 2-MeI and 1.05 μg of 4-MeI. The latter corresponds to a concentration of 105 μg/g in the original caramel color sample.

Table I. Recovery of Standard Additions of 4-MeI to Model System

Sample	Added 4-MeI, μg/g	Recovered 4-MeI, μg/g	Recovery, %
A-1	50	49, 1.7% RSD[a]	98
A-2	50	51, 1.4% RSD[a]	102
A-3	50	50, 1.4% RSD[a]	100
B-1	100	102, 1.5% RSD[a]	102
B-2	100	99, 1.9% RSD[a]	99
B-3	100	99, 1.4% RSD[a]	99

[a] Relative standard deviation (RSD) for three replicate injections.

2-MeI and 1.05 μg of 4-MeI. The latter corresponds to a concentration of 105 μg/g in the original caramel color sample.

RESULTS AND DISCUSSION

The utility of the method was tested by standard addition recovery studies and reproducibility studies involving two analysts.

A standard addition experiment was carried out using high fructose corn syrup as a model system. Additions of 50 and 100 μg of 4-MeI/g were made to 10.00-g aliquots of the syrup, and the aliquots were then analyzed by the method. Each extract was analyzed in triplicate, and the concentrations shown in Table I represent the averages and relative standard deviations for the triplicate analyses. The recoveries of 4-MeI from the model systems were quite good for each concentration. The precision of the replicate injections is as would be expected for an automatic sampler.

Table II. Recovery of Standard Additions of 4-MeI to Caramel Color

Replicate	Concentrations of 4-MeI, μg/g of caramel color: Added 4-MeI, μg/g: 0	50	100	200
1	5	55	105	202
2	5	53	103	200
3	4	53	103	205
4	3	54	104	206
Mean	4.3	53.8	103.8	203.3
Rel SD, %	22.5	1.78	0.92	1.36
Av % recovery		99.1	99.6	99.5

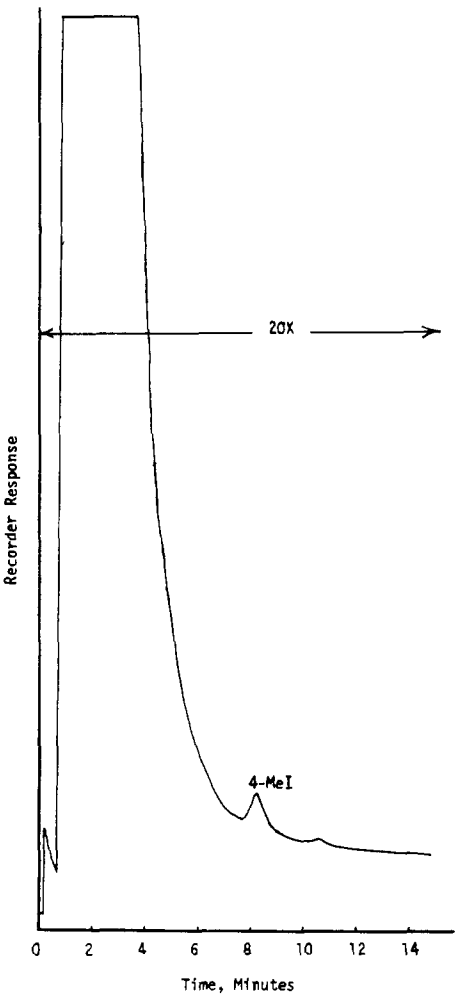

Figure 2. A chromatogram of a 5-μL injection of an extract containing 0.005 μg 4-MeI/5 μL, which corresponds to 0.5 μg of 4-MeI/g of caramel color.

A second standard addition experiment was carried out, this time using an experimental caramel color manufactured under conditions designed to minimize the production of 4-MeI. Additions of 50, 100, and 200 μg of 4-MeI/g were made to 10.00-g aliquots of the caramel color. Four replicate extractions were made of the caramel color itself and of the spiked aliquots. Each extract was analyzed in triplicate and the results are shown in Table II. As for the first experiment, the precision of replicate injections by the autosampler was on the order of 1% relative standard deviation. The means and relative standard

Table III. Replicate Extractions of Caramel Color Samples by Two Analysts

Sample	Analyst A	Analyst B	Mean, RSD[a]
202-1	195	196	199.5, 2.0%
202-2	204	198	
202-3	200	204	
	200, 2.39% RSD[b]	199, 2.1% RSD[b]	
344-1	4	4	4.7, 17.5%
344-2	5	5	
344-3	5	4	
	4.7, 12.4% RSD[b]	4.7, 24.7% RSD[b]	
418-1	83	79	80, 2.4%
418-2	79	78	
418-3	82	81	
	81, 2.6% RSD[b]	79, 1.9% RSD[b]	
423-1	638	652	
423-2	647	639	647, 1.1%
423-3	655	649	
	647, 1.3% RSD[b]	647, 1.1% RSD[b]	
495-1	330	336	
495-2	336	332	336, 1.5%
495-3	343	341	
	336, 1.95% RSD[b]	336, 1.3% RSD[b]	
546-1	98	104	
546-2	106	107	103, 3.5%
546-3	101	100	
	102, 4.0% RSD[b]	104, 3.4% RSD[b]	

[a] Mean and relative standard deviation for all extracts of a caramel color sample. [b] Mean and relative standard deviation (RSD) for triplicate extractions of a caramel color sample by one analyst.

deviations for the replicate extractions are very good, indicating the precision of the method. In addition, once again the recovery data are excellent over the range of concentrations employed.

An experiment designed to test the reproducibility of the method when run by two different analysts was carried out. Six caramel color samples were extracted in triplicate by the two analysts. Of the samples used, four were commercially available beverage-type caramel colors, the fifth was an experimental caramel color manufactured so as to enhance 4-MeI production, and the sixth was an experimental caramel color manufactured so as to minimize the production of 4-MeI. The results of the experiment are shown in Table III. The individual extract concentrations represent the averages of triplicate injections. As for the previous experiments, the precision of the automatic sampler was on the order of 1% relative standard deviation. The data in Table III indicate that the method can be used by different analysts to yield accurate and precise results. This is evident for all samples, over the extremely wide range of concentrations employed, although the relative standard deviations for sample 344 are misleadingly large due to the small values involved.

With the GLC parameters described above, the lower limit of detection of 4-MeI is approximately 0.20 μg/g of caramel color, or 1% of the FAO/WHO limit of 200 mg/kg. Figure 2 is a chromatogram of a 5-μL injection of an extract containing 0.005 μg of 4-MeI/5 μL, which corresponds to 0.5 μg of 4-MeI/g of caramel color.

The method described has been shown to be accurate and precise when used with both commercially available caramel color samples as well as experimental samples to which standard additions were made. It is capable of quantitative recovery of 4-MeI over a wide range of concentrations and can determine 4-MeI at sub-μg/g levels in caramel color. In addition, it meets the criterion of speed mentioned earlier; the time required to make duplicate extractions of caramel color samples is approximately 1.5 h. Thus the method is suitable for routine quality monitoring as well as for research applications.

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