Journal of Chromatography, 87 (1973) 411–418

CHROM. 7029

THE ISOLATION OF 4-METHYLIMIDAZOLE FROM CARAMEL COLOR AND ITS DETERMINATION BY THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY

R. A. WILKS, JR., A. J. SHINGLER and L. S. THURMAN
The Coca-Cola Co., Atlanta, Ga. (U.S.A.)
and
J. S. WARNER
Battelle's Columbus Laboratories, Columbus, Ohio (U.S.A.)
(Received May 28th, 1973)

SUMMARY

The presence of small quantities of 4-methylimidazole (4-MeI) in caramel color has been confirmed by retention time data, and infrared and mass spectroscopy. This paper describes a method for the quantitative extraction of 4-MeI from caramel color, and two chromatographic procedures—thin-layer (TLC) and gas–liquid (GLC) chromatography— for the quantitative determination of 4-MeI in the extracts. The accuracy and precision of the extraction and the chromatographic procedures were tested by analysis of: (a) model caramel color systems; (b) samples of experimental caramel colors of low 4-MeI content; and (c) duplicate samples of caramel color by two participating laboratories. The extraction procedure permits the quantitative recovery of 4-MeI from samples of caramel color; the TLC and GLC analysis procedures provide approximately equal accuracy, with somewhat greater precision afforded by the GLC procedure. The GLC procedure is therefore recommended when precise determinations of 4-MeI concentrations are required; the TLC procedure is recommended for screening studies where a rough estimation of 4-MeI is sufficient.

INTRODUCTION

Commercial caramel color is manufactured by a sugar–ammonia reaction procedure, during which small quantities of substituted imidazoles, including 4-methylimidazole (4-MeI), are formed. The quantities are minimal since the particular reaction conditions which maximize color formation are not conducive to the formation of nitrogenous compounds. However, as part of a study designed to investigate the reaction conditions which control the formation of imidazoles, methodology for the isolation and quantitative determination of 4-MeI in caramel color has been developed.

A solvent extraction procedure has been developed [1,2] for 4-MeI in caramel color which employed both thin-layer (TLC) and gas–liquid (GLC) chromatographic estimations. However, these estimation techniques provided only semiquantitative data, and work was therefore initiated toward development of a quantitative method for 4-MeI in caramel color. This work, based in part on Warner's original efforts, has resulted in a somewhat simpler technique, which has been shown to extract 4-MeI quantitatively from caramel color, and to provide accurate and precise determinations of the amounts of 4-MeI in the extracts.

EXPERIMENTAL

Apparatus

The following apparatus is used: Rotary vacuum evaporator with vacuum source; hot plate and pan for water-bath; 200-ml round-bottom boiling flask; 150-ml beaker; 10-ml volumetric flask; 250-ml separatory funnel, preferably with Teflon* stopcock; 50-ml graduated cylinder; 75-mm powder funnel; 250-ml erlenmeyer flask; 25 mm × 250 mm chromatography column with stopcock; glass-wool; 10-ml sample vials with Teflon-lined caps; 20 cm × 20 cm precoated TLC glass plates with 250-μm layer of silica gel F_{254}.

Reagents

The following reagents —ACS grade or equivalent (where applicable)— are applied: ammonium hydroxide; Celite 545**; chloroform; ethyl alcohol; hydrochloric acid; 4-methylimidazole; sodium bicarbonate, sodium carbonate; sodium hydroxide; sodium nitrite; sulfanilic acid; sulfuric acid.

Standard solutions

A 1000 μg/ml solution of 4-MeI in 0.1 *N* H_2SO_4 is prepared, using 4-MeI (Research Organic/Inorganic Chemical Corp., Sun Valley, Calif., U.S.A.), which has been purified by redistillation (b.p. 92–93°, 0.05 mm Hg). This stock solution is held refrigerated. In preparing standard solutions for TLC and GLC analyses, the stock solution is diluted appropriately and then brought to approximately pH 9 with solid Na_2CO_3. The standard solutions are stable if held refrigerated; however, if they are allowed to remain at room temperature, losses of serious magnitude may occur.

Extraction of caramel color

A basic column packing is prepared by thoroughly mixing 3 g Celite 545 and 2 ml 2 *N* NaOH (1.33 mequiv. NaOH/g Celite 545). A fine glass-wool plug is placed in the bottom of a 25 mm × 250 mm chromatographic column equipped with a stopcock, followed by 5 g of the basic column packing. The packing is firmly tamped to a uniform mass. The caramel color is mixed well by shaking or stirring, and a 10.00-g aliquot is then added to 6.0 g of aqueous 20% (w/v) Na_2CO_3 solution in a 150-ml beaker with thorough mixing. Ten grams of Celite 545 are added to the beaker, the contents are again mixed thoroughly, and are then placed above the basic Celite 545 in the column. The beaker is dry-washed with about 1 g Celite 545, and the washings are

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* A registered trademark of DuPont, Wilmington, Del., U.S.A.

** A registered trademark of Johns-Manville Celite Division, New York, N.Y., U.S.A.

added to the column. A plug of glass-wool is placed above the drywashings and the contents are settled by allowing the column to fall vertically a few centimeters to a padded surface. The contents are tamped firmly to assure uniformity of packing, and the column is eluted with a mixture of chloroform and ethanol (80:20) at a rate of approximately 5 ml/min, until 125 ml* of eluate has been collected.

The eluate is extracted with a 25-ml portion of 0.05 *N* H_2SO_4 and then with a second 10-ml portion of 0.05 *N* H_2SO_4**. The combined aqueous layers are quantitatively transferred to a 200-ml round-bottom boiling flask and concentrated to approximately 5 ml, using a rotary vacuum evaporator operated at 20–40 torr and with the flask maintained at 55° in a water-bath. The concentration must be watched carefully to be sure that no bumping occurs and that the volume is not reduced below 3 ml. The aqueous concentrate is then transferred to a 10 ml volumetric flask (a disposable Pasteur pipet is convenient for this transfer). The boiling flask is rinsed several times with approximately 1-ml portions of distilled water, and the rinsings are added to the volumetric flask until the mark is reached. After mixing thoroughly by several inversions of the flask, the aqueous concentrate is transferred to a suitable sample vial (with a Teflon lined cap) and treated with small portions of solid Na_2CO_3 until CO_2 evolution ceases and pH test paper indicates an alkaline (pH $\geq$ 9) solution***.

TLC analysis

The alkaline aqueous extract may be analyzed by TLC, using glass plates coated with 250-μm layers of silica gel F_{254}, followed by activation at 110° overnight. Duplicate 2.00-μl aliquots of the aqueous extract are spotted on a plate along with 2.00-μl aliquots of standard solutions of 4-MeI containing 100, 150, 200, 250, 300, 350, 400, or 500 μg/ml 4-MeI, respectively. If an extract spot yields a color intensity greater than that of the 500 μg/ml standard solution, the caramel color sample should be re-extracted, using an appropriately smaller aliquot. The plate is developed with ether–chloroform-methanol–ammonium hydroxide (80:20:20:4) until the solvent front has traveled 15 cm. The plate is air-dried and the developed spots are visualized by spraying with a freshly prepared mixture of one part aqueous 0.5% (w/v) sodium nitrite, one part aqueous 0.5% (w/v) sulfanilic acid in 2% (v/v) hydrochloric acid, and two parts aqueous 8% (w/v) sodium bicarbonate. The sodium nitrite and sulfanilic acid solutions are mixed well prior to adding the sodium bicarbonate. Prior to mixing, the three component solutions are stable for six months or more; but once mixed, the spray solution should be used within 2–3 min. The red-orange 4-MeI spot attains maximum intensity in 4–5 min, and remains stable for approximately 30 min thereafter. The amounts of 4-MeI in the alkaline extracts are estimated by comparing the intensities of the 4-MeI spots with those of the standards.

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* Work in these laboratories has indicated that elution of 125 ml at the rate of 5 ml/min (controlled by adjustment of the stopcock) is sufficient to quantitatively extract 4-MeI from 10.00 g caramel color. However, this may be verified by eluting an additional 50-ml portion of solvent and carrying it through the remainder of the procedure.

** This extract should be strongly acidic (pH $<$ 3) by pH test paper. More than 25 ml of 0.05 *N* H_2SO_4 may be required when extracting caramel color samples having high ammonia contents.

*** The acidic concentrate is stable for at least several months when refrigerated. After pH adjustment, interfering decomposition products resulting from other components present may appear within a few days.

GLC analysis

Begg and Grimmett[3] reported a GLC procedure for the separation and identification of imidazoles which used a column of solid support coated with 5% OV-17. They found, however, that imidazoles unsubstituted at the ring nitrogen required conversion to their acyl derivatives prior to analysis. In the present work, 4-MeI is determined without resorting to derivatization by using a strongly alkaline column. A stainless-steel column is rinsed with alcoholic KOH (approx. 5%, w/v), air-dried by vacuum aspirator and packed with 5% Carbowax 20M + 2% KOH coated on 80–90 mesh Anakrom AB (Analabs, North Haven, Conn., U.S.A.). The Anakrom AB

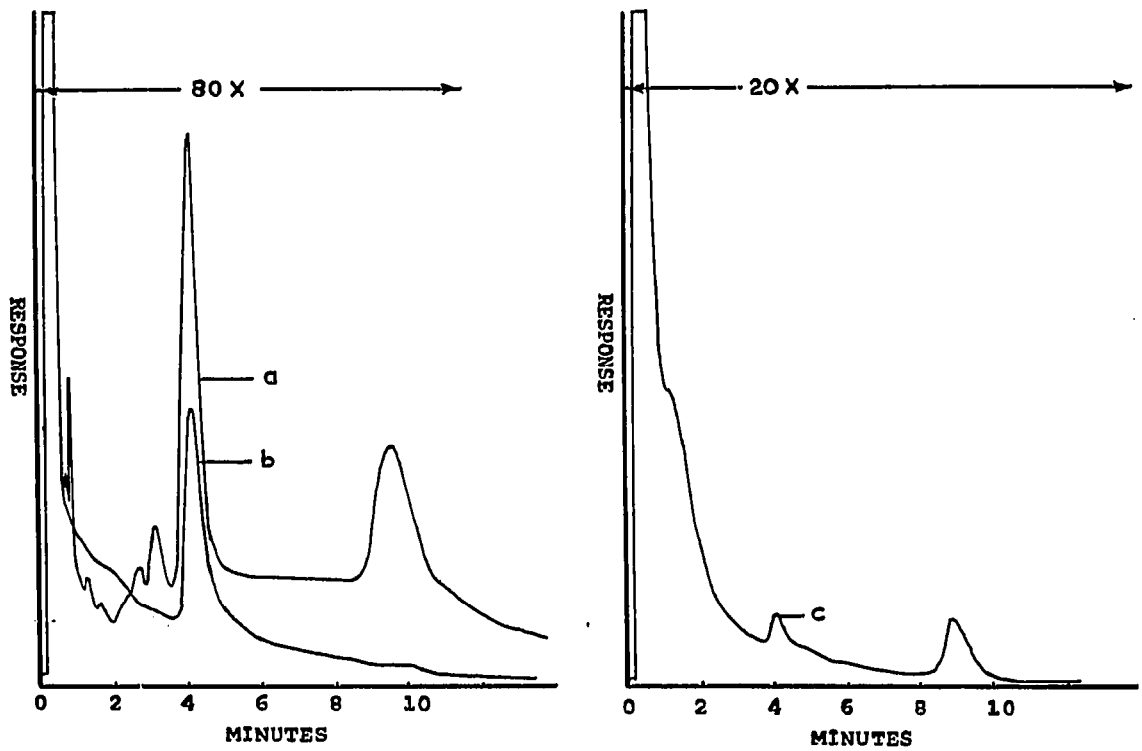

Fig. 1. Gas–liquid chromatograms of extracts of caramel color samples. (a) 4-MeI peak from injection of 5.0 μl of the extract of a caramel color sample containing 250 μg/g 4-MeI. (b) 4-MeI peak from injection of 5.0 μl of 125 μg/ml standard solution. (c) 4-MeI peak from injection of 5.0 μl of the extract of an experimental caramel color sample containing 6 μg/g 4-MeI.

is made more basic by the addition of 2% KOH applied as an alcoholic solution (approx. 5%, w/v), and is then coated with the Carbowax 20M. The column is initially conditioned by programming the temperature from ambient to 190° at 1°/min and holding at 190° overnight. Further conditioning, consisting of injection of several 5.0-μl aliquots of the 1000 μg/ml stock solution (adjusted to pH 9 with solid sodium carbonate), results in a column which yields good separation of the components present and a 4-MeI peak with a minimum of tailing. Fig. 1 shows typical chromato-

grams. The chromatographic parameters used are as follows: column, 4 ft. × 1/4 in. stainless steel*; helium flow-rate, 80 ml/min; column temperature, 180°; injection port temperature, 200°; flame ionization detector temperature, 250°; sample size, 5.0 μk, using the solvent flush technique, with distilled water as the solvent.

The external standard technique is used to quantify the GLC responses from the alkaline extracts. The response data obtained from 5.0-μl aliquots of the standard solutions are plotted *versus* concentration to produce a standard curve. The extract 4-MeI concentrations are then obtained by comparison to the standard curve. When the standard solutions are chromatographed daily over a period of weeks, the standard curves generated will remain linear over the range of concentrations employed, although the slopes may vary somewhat. The standard curve is therefore prepared during each day's runs by interspersing analyses of standard solutions with analyses of the unknown extracts.

RESULTS AND DISCUSSION

The tentative identification of the 4-MeI peak or spot from a chromatographic analysis, based on retention time or R_F data, was confirmed by mass and in-

TABLE I

GLC ANALYSES OF MODEL CARAMEL COLOR SAMPLES CONTAINING STANDARD ADDITIONS OF 4-METHYLIMIDAZOLE

Sample	*4-MeI added (ug/g)*	*4-MeI found (ug/g)***	*σ, rel**	*Mean 4-MeI found (ug/g)****	*σ, rel**	*% recovery*
A-1	25	21.0 ± 0	0	21.1 ± 2.4	0.7	84.4
A-2	25	21.2 ± 1.3	3.9			
B-1	50	46.0 ± 4.9	6.7	47.9 ± 2.4	5.5	95.8
B-2	50	49.8 ± 3.5	4.5			
C-1	100	102.0 ± 5.8	3.6	104.1 ± 2.7	2.9	104.1
C-2	100	106.3 ± 4.7	2.8			
D-1	150	151.3 ± 4.0	1.7	148.0 ± 4.1	3.1	98.7
D-2	150	144.8 ± 12.9	5.6			
E-1	200	196.3 ± 13.3	4.3	193.2 ± 4.0	2.3	96.6
E-2	200	190.0 ± 2.7	1.0			

* σ, rel $= \sigma/\bar{x} \times 100$.

** Mean concentrations and 95% confidence intervals for four replicate determinations.

*** Mean concentrations and 95% confidence intervals for two duplicate extractions, each with four replications.

frared spectroscopy of trapped GLC and TLC eluates. The utility of the method in measuring accurately and precisely the concentrations of 4-MeI in caramel color samples was tested with a series of experiments, described below.

GLC analyses of 4-MeI in model caramel samples

A standard addition experiment was performed by preparing and extracting a series of model caramel colors to which known additions of 4-MeI had been made.

* A 6 ft. × 1/8 in. stainless-steel column, prepared as above, has also been used successfully with 3.0-μl injections, yielding essentially equivalent results.

A liquid sugar syrup was used as the model caramel color, and additions of the 4-MeI stock solution equivalent to 25, 50, 100, 150, or 200 μg/g 4-MeI were made to duplicate 10.00-g portions of the syrup. These duplicate portions were then carried through the extraction procedure, and the aqueous extracts were analyzed in quadruplicate by the GLC method. The results obtained are presented in Table I. The recovery percentages are satisfactory, particularly when the low levels of 4-MeI used and the number of transfers involved are considered. The precision of the analyses is also quite good.

GLC analyses by two laboratories of 4-MeI in experimental caramel color samples

The method was tested further by a similar standard addition experiment run simultaneously by two participating laboratories. A laboratory batch of caramel color was used which had been prepared under conditions designed to minimize 4-MeI production. The results obtained are presented in Table II. Again, the recoveries

TABLE II

GLC ANALYSES BY TWO LABORATORIES OF CARAMEL COLOR SAMPLES CONTAINING STANDARD ADDITIONS OF 4-METHYLIMIDAZOLE

Sample	*4-MeI added (ug/g)*	*4-MeI found (ug/g)***	*Mean 4-MeI found (ug/g)****	*σ, rel** (%)*	*% Recovery*
Laboratory A					
A-1	25	24.1 ± 1.2	22.2 ± 3.1	2.8	88.8
A-2	25	20.3 ± 0.9			
B-1	50	48.0 ± 0.9	48.1 ± 0.9	1.1	96.0
B-2	50	48.2 ± 0.9			
C-1	100	99.1 ± 3.6	99.0 ± 4.5	2.7	99.0
C-2	100	98.9 ± 5.3			
D-1	150	148.7 ± 4.6	147.4 ± 5.0	2.1	98.3
D-2	150	146.1 ± 5.4			
E-1	200	200.0 ± 6.2	199.2 ± 4.5	1.4	99.6
E-2	200	197.9 ± 3.0			
F-1	0	1.6 ± 0.5	1.5 ± 0.4	2.4	—
F-2	0	1.3 ± 0.3			
Laboratory B					
A-1	25	27.8 ± 4.4	24.7 ± 3.9	10.9	98.8
A-2	25	21.6 ± 4.0			
B-1	50	47.8 ± 7.2	48.0 ± 6.6	4.3	96.0
B-2	50	48.2 ± 6.2			
C-1	100	105.1 ± 3.0	103.0 ± 3.7	2.6	103.0
C-2	100	100.8 ± 5.2			
D-1	150	160.1 ± 10.8	160.9 ± 7.5	1.9	107.3
D-2	150	161.2 ± 5.8			
E-1	200	206.6 ± 9.9	206.9 ± 3.4	1.5	103.0
E-2	200	205.4 ± 7.2			
F-1	0	9.6 ± 3.5	9.4 ± 3.7	3.2	—
F-2	0	9.1 ± 3.9			

* σ, rel $= \sigma/\bar{x} \times 100$.

** Mean concentrations and 95% confidence intervals for four replicate GLC injections of each extract.

*** Mean concentrations and 95% confidence intervals for duplicate extractions, each with four replicates.

are satisfactory and the precision, both among the replicate analyses of an extract and between the extracts of duplicate samples, is acceptable. In addition, the interlaboratory agreement is quite satisfactory.

GLC analyses by two laboratories of 4-MeI in caramel color samples

The utility of the method was tested by dividing equally each of two samples of caramel color and distributing the portions to the two participating laboratories. Each sample was extracted six times by each laboratory, and the precision between the replicate extractions was calculated. The data from these extractions are presented in Table III. Once again, the precision between replicate GLC analyses of an extract and between replicate extracts is good, and the interlaboratory agreement is acceptable.

TABLE III

GLC ANALYSES OF CARAMEL COLOR SAMPLES BY TWO LABORATORIES

	Laboratory A		*Laboratory B*	
Sample	*Mean 4-MeI (ug/g)***	*σ, rel** (%)*	*Mean 4-MeI (ug/g)***	*σ, rel** (%)*
137-1	218.8 ± 17.5	5.0	249.3 ± 6.3	2.4
137-2	203.3 ± 6.0	3.2	227.9 ± 2.7	1.1
137-3	205.3 ± 13.1	4.0	205.0 ± 4.4	2.0
137-4	198.6 ± 8.5	3.5	220.3 ± 5.3	2.3
137-5	211.1 ± 14.1	4.2	253.8 ± 3.8	1.4
137-6	207.6 ± 10.0	3.9	230.2 ± 6.6	2.7
	207.5 ± 7.3	3.4	228.0 ± 8.9	7.9
138-1	448.3 ± 17.0	2.4	476.7 ± 9.6	1.9
138-2	433.2 ± 15.2	2.2	476.0 ± 9.6	1.9
138-3	439.0 ± 11.0	1.6	426.9 ± 17.8	4.0
138-4	468.4 ± 28.1	3.7	460.8 ± 12.9	2.6
138-5	444.7 ± 8.6	1.1	435.2 ± 6.6	1.5
138-6	433.4 ± 16.9	2.4	425.8 ± 7.2	1.6
	444.3 ± 11.1	2.4	445.2 ± 25.3	5.3

* σ, rel $= \sigma/\bar{x} \times 100$.

** Mean concentrations and 95% confidence intervals for four replicate GLC injections of each extract.

GLC and TLC analyses of 4-MeI in experimental caramel color samples

A comparison of the GLC analysis technique and the TLC analysis technique was provided by the extraction of a series of experimental caramel color samples. The alkaline extracts were analyzed both by TLC and GLC and the results obtained appear in Table IV. The two methods of analysis appear to provide essentially the same values, but the superior precision of the GLC technique is evident.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the aid and advice of Mr. A. B. Allen and Dr. T. Radford of the Corporate Technical Division of The Coca-Cola Co. Acknowledgement is also due to Dr. A. V. Clark of the Corporate Technical Division of The

TABLE IV

GLC AND TLC ANALYSES OF EXPERIMENTAL CARAMEL COLOR SAMPLES

Sample	*4-MeI by TLC (ug/g)**	*4-MeI by GLC (ug/g)***	*TLC/GLC*
A	62.5 ± 12.5	65 ± 6	0.961
B	100 ± 12.5	108 ± 7	0.926
C	112.5 ± 12.5	109 ± 10	1.027
D	125 ± 12.5	124 ± 9	1.008
E	150 ± 12.5	140 ± 9	1.071
F	87.5 ± 12.5	76 ± 6	1.151
G	137.5 ± 12.5	139 ± 6	0.989
H	87.5 ± 12.5	92 ± 7	0.951
I	50 ± 12.5	58 ± 6	0.862

* Average and range for four independent judgements of spot intensity.

** Concentrations and 95% confidence intervals calculated from the average of four replicate injections.

Coca-Cola Co. and Mrs. C. B. Martin (formerly of The Coca Cola Co., present address: Environmental Protection Agency, Washington, D.C.), who participated in the early stages of the work; and to Mr. R. E. Wyant and Mr. P. C. Behrer, of Battelle's Columbus Laboratories, who assisted in the experimental work.

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