cent reduction ranged from 60 to 70% for total TDE, 59 to 70% for total DDT, and 71 to 74% for total endosulfan.

While the percentage reduction of pesticides is significant, further investigation of process parameters might establish optimal conditions for even further reductions. For example, factors such as water temperature, water/solids ratio, rest time prior to freezing and/or extraction, and product temperature during sublimation may relate to ease of pesticide removal.

It is also likely that, in addition to freeze drying, other manufacturing processes might contribute to pesticide reduction. This could be expected, particularly for new or existing processes which involve moistening tobacco to moderately high levels followed by drying at elevated temperatures. Results of this study should be important to future approaches aimed at reducing pesticide residues in tobacco.

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LITERATURE CITED

Bundesgestzblatt 655, Teil 1, 21997-A, Ausgegeben Zun Bonn am., Dec 10, 1966, No. 53; amended Jan 18, 1972.
Domanski, J. J., Sheets, T. J., *Tob. Sci.* **17**, 55 (1973).
Domanski, J. J., Sheets, T. J., *Beitr. Tabakforsch.*, in press (1975).
Domanski, J. J., Sheets, T. J., Nelson, L. A., *Tob. Sci.* **16**, 157 (1972).
Gibson, J. R., Jones, G. A., Dorough, H. W., Lusk, C. I., Thurston, R., *Pestic. Monit. J.* **7**, 205 (1974).
Guthrie, F. E., *Beitr. Tabakforsch.* **7**, 195 (1973).
Guthrie, F. E., Sheets, T. J., *Tob. Sci.* **14**, 44 (1970).
Johnson, W. H., Proceedings of the International Tobacco Scientific Congress, Hamburg, Vol. V, 1970, p 142.
Johnson, W. H., *Tob. Sci.* **17**, 110 (1973).
Zabik, M. E., Dugan, L., Jr., *J. Food Sci.* **36**, 87 (1971).

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Quantitative Determination of 4-Methylimidazole as 1-Acetyl Derivative in Caramel Color by Gas–Liquid Chromatography

A gas–liquid chromatographic (glc) method has been developed for the quantitative determination of 4-methylimidazole (4-MeI) in caramel color. Prior to glc the 4-MeI was converted to a 1-acetyl derivative. The identity of 4-MeI in caramel color extracts has been confirmed by thin-layer chromatography, retention time data on two stationary phases of differing polarity, and mass spectrometry. The quantitation has been achieved by using 2-methylimidazole as internal standard. The accuracy and precision of the method were also tested, by recovery experiments and replicate analyses on four different commercial caramel colors. The acylation step has also been studied, by nuclear magnetic resonance.

Caramel color is a frequently used food additive in various food commodities, *e.g.*, carbonated beverages, baked products, and dry mixes, as a coloring and flavoring agent. Caramel color is the amorphous dark brown material resulting from the controlled heat treatment of various food grade carbohydrates such as dextrose, invert sugar, lactose, malt syrup, molasses, or starch hydrolysates. There are various acids, alkalis, and salts which may be used to assist caramelization. If ammonium hydroxide or ammonium salts are used as a process aid, the color is specified "prepared by the ammonia process." During this procedure small quantities of substituted imidazoles can be formed, *e.g.*, 4-methylimidazole (4-MeI) from pyruvaldehyde and ammonia in aqueous solution, according to Grimmett and Richards (1965). Commercial caramel colors made by the ammonia process contain up to several hundred milligrams of 4-MeI/kg.

The presence of 4-MeI in the caramel color is undesirable because of its toxicity. The toxic effect of various imidazoles on mice has been investigated by Nishie *et al.* (1969).

In view of these facts, further information about the levels of 4-MeI in caramel colors is needed as is also a suitable method for the determination of this compound. Such a method for the isolation and determination of 4-MeI in caramel color has been developed recently by Wilks *et al.* (1973). Their method is based on extraction and direct chromatography of the 4-MeI in free form and subsequent quantitation by an external standard technique. The gas chromatographic step was carried out on an alkali-treated polar stationary phase. It was necessary to prepare a new standard curve for each day's runs. In the present study another technique has been tried, involving derivatization and application of a suitable internal standard.

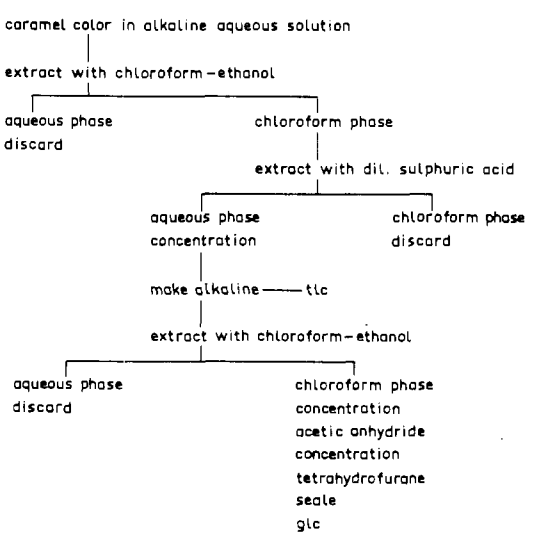
It has been recently noted that Begg and Grimmett (1972) have successfully separated several imidazoles as their 1-acetyl derivatives by glc. It seemed feasible that such a derivative could facilitate the determination of 4-MeI in caramel color extracts, too. For the quantitative assessment, 2-methylimidazole (2-MeI) was chosen as internal standard. It was found that this compound was absent in the caramel colors examined in this work and also that 2-MeI acetylated simultaneously with 4-MeI and the derivatives separated well on a common, polar stationary phase (STAP).

EXPERIMENTAL SECTION

Extraction and Derivatization. Five different commercial caramel colors manufactured by the ammonia process were examined. The caramel color (20.0 g) was weighed into a 100-ml beaker and dissolved in 10 ml of 10% sodium carbonate solution. The solution was transferred to a 250-ml separatory funnel. Internal standard (4.00 ml) solution (1.00 mg of 2-MeI/ml of chloroform–ethanol (80:20, v/v)) and 100 ml of the same solvent were added. The mixture was shaken vigorously for 1 min and then allowed to stand for about 15 min. The chloroform phase was collected in a 500-ml erlenmeyer flask. The extraction step was repeated twice with 100 + 50 ml and the chloroform phases were collected in the same flask. The chloroform phase was dried over 20 g of anhydrous potassium carbonate under magnetic stirring for 30 min. The mixture was filtered and the drying agent was washed with methylene chloride. The clear yellow filtrate was extracted with 0.05 *N* sulfuric acid 25 + 10 ml. The acidic water phases were collected in a 250-ml round-bottomed flask and concentrated to about 5 ml, using a rotary vacuum evaporator operated at 50–60° in the water bath. The concentration must be watched carefully to ensure that no bumping occurs and that the volume is not reduced below 3 ml. The aqueous concentrate was then transferred to a 10-ml volumetric flask. The boiling flask was rinsed several times with water and the volumetric flask was filled with water up to the mark and the contents then mixed thoroughly. The aqueous solution was treated with small portions of solid sodium carbonate until pH ≥9 was reached. An alkaline aqueous extract containing no 2-MeI was analyzed by thin-layer chromatography (tlc) according to Wilks *et al.* (1973). The 2-MeI was omitted here because of interference with 4-MeI on the tlc. The presence of 4-MeI was verified by comparing the spots from extracts with those of the standards.

The alkaline solution (*cf.* above) was extracted with 4 × 10 ml portions of chloroform–ethanol. The chloroform phases were collected in a 50-ml round-bottomed flask and evaporated to dryness using a rotary evaporator. The flask was maintained at 40° in a water bath. One milliliter of acetic anhydride was added to the residue in the flask. The flask was capped with a glass stopper and the contents mixed ultrasonically. The solution was concentrated nearly to dryness by using the rotary evaporator. The residue was dissolved in 0.2 ml of tetrahydrofuran and transferred to 1-ml vials, and the vials were sealed. The sample was then ready for glc (*cf.* the schematic diagram of extraction and derivatization (Scheme I)).

Scheme I



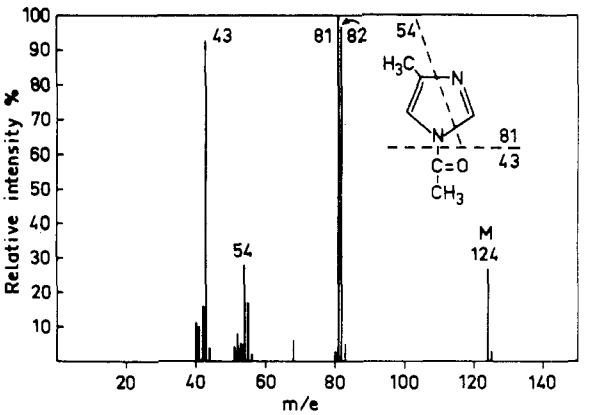


Figure 1. Mass spectrum of 1-acetyl-4-methylimidazole.

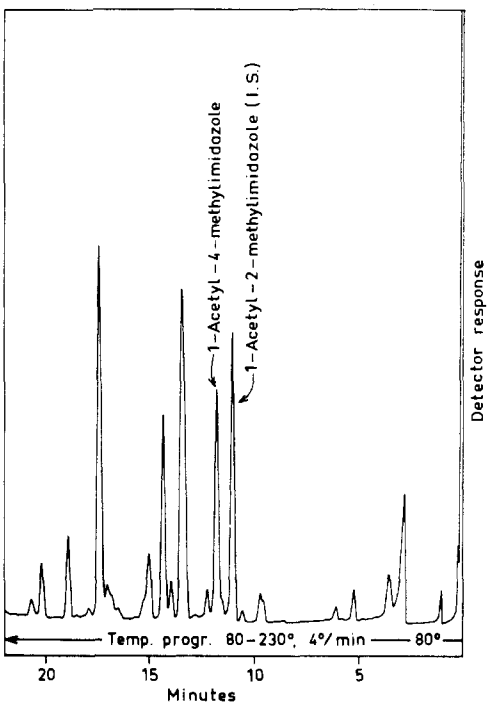


Figure 2. Gas chromatogram of derivatized caramel color sample; column: 3% STAP on 100–120 mesh Chromosorb W (DMCS), 6 ft × ⅛ in. i.d. glass.

Mass Spectrometry (Ms) and Nuclear Magnetic Resonance (Nmr) Studies. The ms analyses were performed on a Varian Model CH 7 combined with a Varian Model 1740 gas chromatograph and Varian Spectrosystem 100 MS. 4-MeI was identified in the caramel color extract (a run without internal standard) by combined glc-ms. The gas chromatograph was equipped with a 6 ft × ⅛ in. glass column packed with 3% OV-1 on 100–120 mesh Chromosorb W (DMCS). The glc column was operated under the following parameters: helium carrier gas flow rate, 20 ml/min; initial column temperature, 30°; final temperature, 230°; temperature programming rate, 4°/min; inlet 200°. The fragmentation of the 1-acetyl-4-methylimidazole is shown in Figure 1.

The nmr studies were undertaken to examine the course of the acetylation stage when 2-MeI and 4-MeI were present simultaneously. The measurements were performed on a Varian HA-100 instrument. Equivalent amounts of these two compounds were dissolved in dichloromethane. The chemical shifts of the protons H_4 and H_5 originating from 2-MeI and the protons H_2 and H_5 from 4-MeI were measured. An insignificant amount of acetic anhydride was

Table I. 4-Methylimidazole Content (Milligrams/Kilogram) of Various Caramel Colors

Caramel color	No. of determinations	Mean values and mean dev.	S.D.
A	11	231 ± 8	4.5
B_1	2	229 ± 0	
B_2	5	303 ± 7	3.9
C	4	25 ± 2	
D	2	47 ± 7	

Table II. Recovery of 4-Methylimidazole Added to Various Caramel Colors

Caramel color	No. of determinations	Added, mg	Recovery, % Range	Recovery, % Mean
A	2	4.00	90–102	96 ± 6
A	2	5.93	99–102	100 ± 1
A	4	4.08	90–104	96 ± 6
B	3	5.93	88–104	94 ± 7
C	3	0.94	87–97	92 ± 3
C	5	1.02	93–106	101 ± 4

added to the solution and the nmr spectrum was recorded again. The chemical shifts and integral tracing of all of the protons (H_2, H_4, and H_5) from the acetyl derivatives and also from the parent compounds were recorded and the positions were established according to Reddy *et al.* (1963) who have observed that the shifts of the protons considered in the 2-MeI and 4-MeI were changed by the acetylation reaction. The acetylation reaction was further studied by the continuous supply of acetic anhydride until excess.

Determination. The glc analyses were carried out on a Varian Model 2100 gas chromatograph equipped with flame ionization detector. Retention times and peak areas were measured with a Varian Model 476 electronic integrator. The column was a glass 6 ft × ⅛ in. i.d. U tube packed with 3% STAP on 100–120 mesh Chromosorb W (DMCS). The nitrogen gas flow rate was 20 ml/min and the air and hydrogen flow rates were 300 and 25 ml/min, respectively. The injector and detector temperatures were 200 and 250°, respectively; initial column temperature, 80°; final temperature, 230°; temperature programming rate, 4°/min. Figure 2 shows a typical gas chromatogram of a derivatized caramel color extract. The retention times of 2-MeI and 4-MeI were 10.7 and 11.5 min, respectively. The internal standardization method was used to convert peak areas to amounts of 4-MeI in the caramel colors. The calibration curve was prepared by derivatization and chromatography of 5, 6, 10, 15, and 20 mg of 4-MeI and 10 mg of 2-MeI (to each). Weight ratios of 4-MeI/2-MeI as x were plotted *vs.* average peak area ratios 4-MeI/2-MeI as y. The relative response factor for 4-MeI was 0.77.

Quantitation. The following formula was used

$$[\text{4-MeI}]\ (\text{mg/kg}) = \frac{(y - a)W_{\text{I.S.}}\,1000}{W_S\,b}$$

where a = graph's intercept on the y axis, b = slope of the graph, *i.e.* the relative response, $W_{\text{I.S.}}$ = weight of internal standard added, in milligrams, W_S = weight of the sample, in grams, and y = (counts for the 4-MeI peak)/(counts for the 2-MeI peak).

RESULTS AND DISCUSSION

The nmr investigations showed that the formation of the acetyl derivative is almost instantaneous and also that 2-MeI and 4-MeI were derivatized simultaneously. The acetyl derivatives were stable for several days besides giving rise to symmetrical, triangular-shaped peaks as well when chromatographed (see Figure 2). The derivatization facilitated the direct identification of the 4-MeI by combined glc–ms. The disadvantages associated with the alkaline water solutions were avoided in this manner.

The 2-MeI and 4-MeI peaks were well separated on the polar phase STAP. Other stationary phases were also examined, *e.g.*, OV-1 and OV-225. These phases gave unsatisfactory separation, however. A linear relationship between peak areas and weight ratios for 4-MeI/2-MeI was obtained over the range studied (0.7–1.9 for peak areas and 0.5–2.0 for weight ratios). The 2-MeI should be present in about the same concentration as the 4-MeI measured in the caramel colors in order to achieve maximum accuracy. It is therefore necessary to run an analysis with the recommended internal standard supply (see procedure) for the rough estimation of the 4-MeI level and then adjust the amount of the internal standard, giving a weight ratio of about 1 (0.5–2.0) for 4-MeI/2-MeI.

The precision was determined by replicate analyses which are compiled in Table I. The recoveries were studied by addition of known amounts of 4-MeI to different caramel colors. The results obtained are presented in Table II.

The sensitivity of the method presented in this work was tested on liquid sugar syrup as a model caramel color system. The smallest amount of 4-MeI which could be determined by this method was about 10 mg/kg.

LITERATURE CITED

Begg, C. G., Grimmett, M. R., *J. Chromatogr.* **73**, 238 (1972).
Grimmett, M. R., Richards, E. L., *J. Chem. Soc.*, 3751 (1965).
Nishie, K., Waiss, A. C., Jr., Keyl, A. C., *Toxicol. Appl. Pharmacol.* **14**, 301 (1969).
Reddy, G. S., Mandell, L., Goldstein, J. H., *J. Chem. Soc.*, 1414 (1963).
Wilks, R. A., Jr., Shingler, A. J., Thurman, L. S., Warner, J. S., *J. Chromatogr.* **87**, 411 (1973).

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