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QUANTITATIVE ION-PAIR EXTRACTION OF 4(5)-METHYLIMIDAZOLE FROM CARAMEL COLOUR AND ITS DETERMINATION BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure for quantitative ion-pair extraction of 4(5)-methylimidazole from caramel colour using bis(2-ethylhexyl)phosphoric acid as ion-pairing agent has been developed. Furthermore, a reversed-phase ion-pair liquid chromatographic separation method has been established to analyse the content of 4(5)-methylimidazole in the extracts. A rapid and adequate separation was achieved on a column of Nucleosil 5 C_8 eluted with methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5) containing 0.005 *M* sodium dodecanesulphonate.

This method of determination is superior in speed and repeatability to, and at higher contents of 4(5)-methylimidazole gives a better accuracy than, the World Health Organization method currently official in Denmark. The limit of detection is estimated at 4 μg/g.

INTRODUCTION

Caramel colours are among the most widely used food and drug colouring matters. Commercial caramel colour is mainly manufactured by a sugar–ammonia or by a sugar–ammonia–sulphite reaction procedure, during which imidazole and pyrazine derivatives are formed. The content of 4(5)-methylimidazole (4-MeI) has attracted special attention due to its possible toxicity. At the present time, as a precautionary measure, the World Health Organization has specified the acceptable limit of 4-MeI as 200 ppm based on a caramel colour having a colour intensity of 20,000 European Brewery Convention (EBC) units[1].

Since caramel colour is produced on a large scale, a test for 4-MeI is often done

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and a fast and reliable method is therefore required. So far several methods and many improvements concerning both the extraction and the final determination have been published. The official method in Denmark[1] is based on the work of Wilks *et al.*[2] and involves solvent extraction of a semi-dry mixture of the sample and diatomaceous earth followed by a gas chromatographic (GC) analysis. The extraction step was subsequently changed by Wilks *et al.*[3]. Other isolation techniques are also used, such as ion exchange[4–6] and solvent extraction[7–9]. The analysis of the extract has also been the subject of modifications, for instance through the introduction of 2-methylimidazole as internal standard[3], the conversion into the acetyl derivative before GC[5,7], the use of a nitrogen specific detector[8] or application of reversed-phase ion-pair liquid chromatography[9]. None of the modifications, however, constitutes decisive improvement. For instance, the altered procedure of Wilks *et al.*[3] was found to be subject to interference from an unknown extracted from some batches of caramel colours, biasing the internal standard[10]. Furthermore, none of the published methods was found to have a dynamic range of determination exceeding 200 μg/g of 4-MeI.

All of the above methods are very time-consuming. In particular, when a high accuracy is needed, the workable methods require an analysis time equivalent to not more than four samples per person per day. This problem is mainly caused by insufficient extraction, justifying a closer examination. This paper describes an ion-pair extraction procedure and a high-performance liquid chromatographic (HPLC) separation and their validation for the determination of 4-MeI.

EXPERIMENTAL

Apparatus

Concentrations of 4-MeI in aqueous phases were determined by absorbance measurements at 215 nm using a Beckman Acta III spectrophotometer. pH values of the aqueous phases were read from a Radiometer Model PHM 64 pH-meter.

The liquid chromatograph comprised a Kontron Model 410 LC pump, equipped with a Kontron Model 811 pulse damper, a Rheodyne Model 7125 injection valve and a Pye Unicam LC–UV detector. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder, and retention and area data were measured and processed by means of a Hewlett-Packard Model 3353A laboratory data system.

The gas chromatograph was a Hewlett-Packard Model 5840A equipped with a flame ionization detector and a Hewlett-Packard Model 7672A automatic sampler. Chromatograms were recorded on the plotting integrator of the gas chromatograph, but the peak heights were measured and contents calculated manually.

Chemicals and reagents

4-MeI was obtained from Fluka (Buchs, Switzerland). Bis(2-ethylhexyl)-phosphoric acid (DEHPA) was obtained from BDH (Poole, Great Britain). DEHPA was purified and chloroform was freed from ethanol, both by repeated extraction with 0.1 *M* phosphoric acid. Chloroform as well as all the other reagents were of analytical grade and were obtained from E. Merck (Darmstadt, G.F.R.). Phosphate buffers had an ionic strength of 0.2 *M* and contained potassium as the only cation.

Information about the caramel colours investigated is provided in Table I. The isoelectric points were determined according to White and Munns[11], whereas the

TABLE I

THE CARAMEL COLOURS INVESTIGATED

Sample no.	*Manufacturing procedure*	*Isoelectric point, pH*	*Colour intensity (EBC units)*
1	Ammonia process		43,340
2		4–5	46,660
3		6	32,000
4		4–5	20,000
5		5	36,000
6			29,340
7	Ammonia–sulphite process	1.5	20,660
8		1.5	31,340
9		1.5	26,000
10		1.5	46,660
11		1.5	43,340
12		1.5	48,000
13		1.5	45,340
14		1.5	30,000

colour intensities were measured in EBC units in accordance with the standard of the European Brewery Convention[12].

Ion-pair extraction

A 2.50-g amount of caramel colour was diluted with 15 ml of 0.2 *M* phosphate buffer, pH 6.0, in a 20-ml measuring cylinder. After mixing, the pH was adjusted to 6.0 by dropwise addition of a potassium hydroxide solution. Finally the cylinder was filled to the 20-ml mark with the phosphate buffer.

Four millilitres of this sample solution (equivalent to 0.5 g caramel colour) were extracted in a screw-capped centrifuge-tube with 4.00 ml of 0.1 *M* DEHPA in chloroform by shaking for 0.5 min. After separation by centrifuging, 3.00 ml of the chloroform phase were transferred to a new centrifuge-tube containing 3.00 ml of 0.1 *M* phosphoric acid. By shaking for 0.5 min the content of 4-MeI was re-extracted into the aqueous phase, which after separation by centrifuging is ready for the final determination.

The resulting aqueous phase of the back-extraction contains three quarters of the 4-MeI content originally present in the amount of caramel colour sampled. The ion-pair extraction procedure is illustrated in Fig. 1.

Ion-pair chromatography

Stainless-steel columns (120 × 4.6 mm I.D.) from Knauer (Berlin, G.F.R.) were packed with Nucleosil 5 C_8 and 5 C_{18} (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.) according to a previously described procedure[13]. The efficiency of the columns, expressed as the number of theoretical plates, *N*, measured for naphthalene when eluted by 80% and 90% methanol, respectively, in water at a flow-rate of 1 ml/min, was 9000 for the C_8 and the C_{18} column alike.

The eluent was methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5), and sodium dodecanesulphonate was added as counter ion at a con-

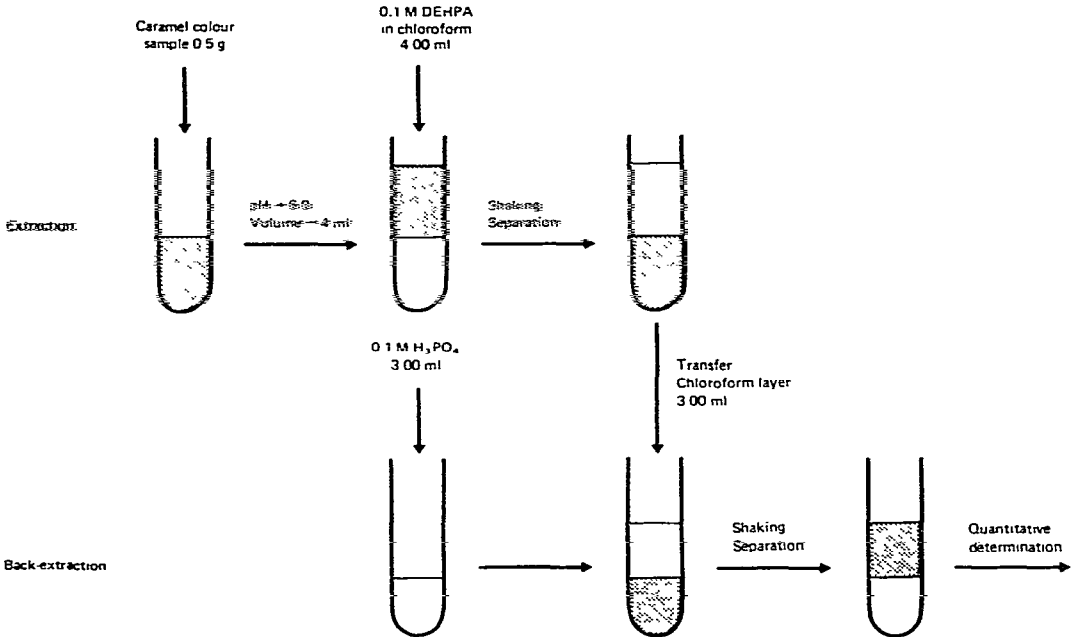

Fig. 1. Ion-pair extraction procedure for quantitative separation of 4-MeI from caramel colour.

centration of 0.005 *M*. A 20-μl volume of the resulting aqueous phase from the back-extraction was injected.

Official method

The extraction was performed according to the official standard[1] modified by using two re-extractions with 10 ml sulphuric acid instead of one. A glass column (1800 × 2.0 mm I.D.) was packed with 5% Carbowax 20M and 2% potassium hydroxide on Chromosorb W AW DMCS (80–100 mesh) (Macherey, Nagel & Co.). The temperatures of the injector, column and detector were 240, 180 and 240°C respectively. The carrier gas was nitrogen at a flow-rate of 30 ml/min. A 2.5-μl volume of sample solution was injected.

RESULTS AND DISCUSSION

Ion-pair extraction

The determination of 4-MeI in caramel colour requires a prior isolation step due to the nature of the colouring matter. The hydrophilic character of 4-MeI leads to a low efficiency of extraction with most organic solvents, making a quantitative isolation difficult. Using an ion-pair technique, particularly in combination with adduct formation, a more hydrophobic product can be obtained.

Working with a similar problem, Modin and Johansson[14] used DEHPA for the isolation of aminophenols and amino alcohols as ion pairs. But even though the pK_a value of 4-MeI is known to be 7.6[15], it was not possible to apply the technique to 4-MeI on the basis of the general procedures proposed by Modin[16]. It was necessary

to determine experimentally the optimum pH for the extraction in order to achieve full recovery with one extraction only. This was done by measuring the net distribution ratio, *D*, of 4-MeI between the organic and aqueous phases using a standard solution of 4-MeI extracted at different pH values and determining the contents spectrophotometrically. The resulting relationship between the logarithm of *D* and pH can be seen in Fig. 2. The extraction with 0.1 *M* DEHPA in the organic phase is virtually complete at pH 6.0 in the aqueous phase. Under these conditions 4-MeI is assumed to form an (1 + 1) ion pair with DEHPA combined with an adduct consisting of 2 moles of DEHPA[17]. This means that groups containing 48 alkyl carbon atoms in all are coupled to the cation, thus allowing full recovery by only one extraction into chloroform. Furthermore, this procedure has the further advantage of yielding a clean extract, due to the selectivity in the co-extraction and the detention of the counter ion in the organic phase during the back-extraction.

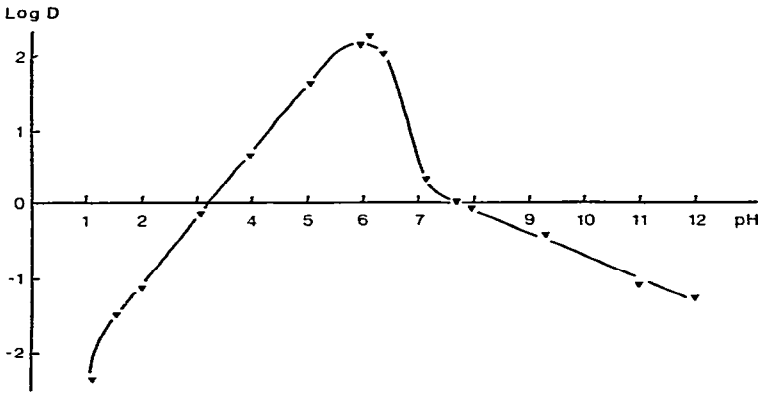

Fig. 2. Relation between logarithm of the net distribution ratio and pH for 4-MeI. The extractions were performed with equal volumes, the organic phases initially comprising 0.1 *M* DEHPA in chloroform and the aqueous phases comprising 4-MeI in a phosphate buffer.

Ion-pair chromatography

The use of an ion-pair technique for the chromatography of a hydrophilic and ionic substance such as 4-MeI is an obvious approach. Davis and Hartford[9] used heptanesulphonate as the ion-pairing agent and an octadecylsilyl bonded silica as support. In our view the chromatographic system should in this case be based on dodecanesulphonate as counter ion and octylsilyl bonded silica as support, according to a study of Helboe and Thomsen[18]. Using Nucleosil 5 C_8 as the support, a suitable composition of the eluent was found to be methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5) with 0.005 *M* sodium dodecanesulphonate.

The separation could also be carried out on octadecylsilyl bonded silica, for instance Nucleosil 5 C_{18}, but the methanol content of the eluent could not be increased, due to interference by a peak from chloroform with which the aqueous phase is saturated during the extraction. The chromatographic behaviour of 4-MeI and chloroform in the systems present can be seen in Fig. 3. The difference leading to intersection of the curves in the range of interest is due to different retention mechanisms of the two substances. 4-MeI seems to be retained partly as an ion pair by a

reversed-phase mechanism and partly by a cation-exchange mechanism. The non-ionic, hydrophobic substance chloroform seems to be chromatographed according to a pure reversed-phase mechanism. The different retention mechanisms are influenced to dissimilar extents by changes in the elution strength of the eluent, leading to intersections of the curves. Additionally, Fig. 3 shows that octylsilyl bonded silica is the more powerful of the two supports with respect to selectivity of the two substances in the range investigated.

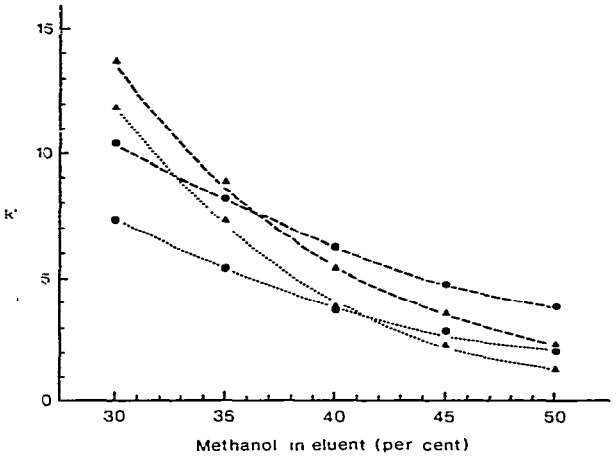

Fig. 3. The behaviour of 4-MeI (▲) and chloroform (●) on Nucleosil C_8 (------) and C_{18} (— — —) columns, expressed by their capacity factors, k', as a function of the percentage of methanol in the eluent containing 0.05 *M* potassium dihydrogen phosphate and 0.005 *M* dodecanesulphonate.

The detection wavelength was chosen on the basis of the absorption spectrum of 4-MeI dissolved in the eluent; the maximum absorption was found at 215 nm. Chromatograms of caramel colours manufactured by the ammonia and by the ammonia–sulphite process respectively are depicted in Fig. 4.

Linearity, recovery and detection limit

The linearity of the detector response and the capability of the extraction procedure were investigated at the same time. Samples of caramel colour from the same batch and equivalent to 1/4, 1/2, 1, 3/2 and 2 times the usual quantity were extracted and analysed according to the described procedures. The results obtained are shown in Table II; a regression analysis showed a small intercept and a satisfactory correlation coefficient.

The recovery of 4-MeI from caramel colour was investigated using a standard amount with different additions of 4-MeI. The results obtained are in Table III. On the basis of these results, which demonstrate an excellent linearity and a quantitative recovery, it was decided to perform the quantitations using standard solutions of one concentration only.

The detection limit of the ion-pair method was investigated by several consecutive determinations on a sample containing a small amount of 4-MeI. From the

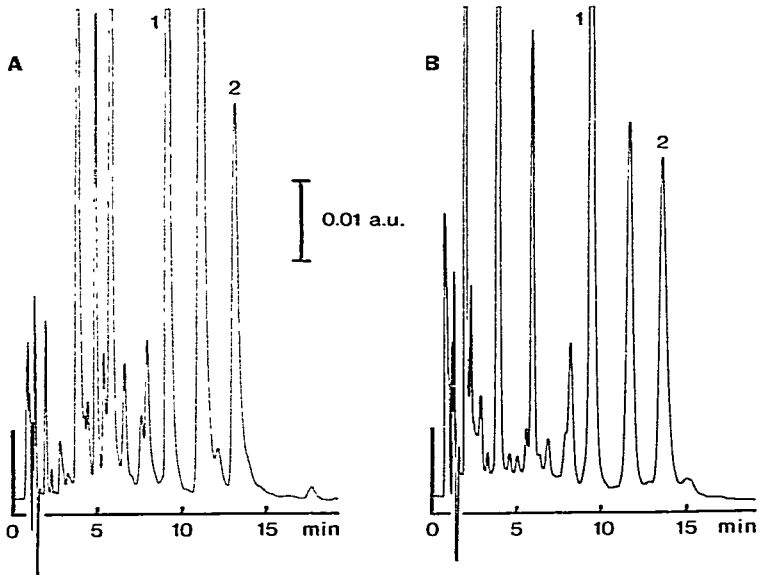

Fig. 4. Chromatograms of caramel colours manufactured by the ammonia process (A) and by the ammonia–sulphite process (B). Support: Nucleosil 5 C_8. Eluent: methanol–0.2 *M* potassium dihydrogen phosphate–water (32.5:25:42.5) containing 0.005 *M* dodecanesulphonate; flow-rate 1 ml/min. Detection: 215 nm. Peaks: 1 = chloroform; 2 = 4-MeI (A, 170 μg/g; B, 142 μg/g).

TABLE II

LINEARITY OF EXTRACTION AND CHROMATOGRAPHIC DETERMINATION FOR DIFFERENT QUANTITIES OF THE SAME CARAMEL COLOUR

Linear regression analysis: correlation coefficient, $r = 0.999$; intercept $= -0.82$.

Sample size (g)	*4-MeI found (μg)*
0.120	9.0
0.241	18.5
0.481	41.8
0.722	61.7
0.963	79.5

results obtained the detection limit was estimated as three times the standard deviation of the results, 4 μg/g for a single determination.

Repeatability

The repeatability of the ion-pair method was compared with that of the official method[1] by carrying out ten subsequent extractions and quantitations according to both methods and using the same caramel colour sample. The results obtained are in Table IV, from which it appears that the ion-pair method surpasses the official method in respect of the repeatability. Concerning the speed of the methods, the ion-pair method is about three times faster than the official one.

TABLE III

RECOVERY OF 4-MeI FROM SPIKED CARAMEL COLOUR SAMPLES AFTER EXTRACTION FOLLOWED BY CHROMATOGRAPHIC DETERMINATION

4-MeI (μg)		*Recovery (%)*
Added	*Found*	
0	42	
13	55	100.0
53	96	101.0
132	178	102.3
265	313	102.0
529	593	103.8

TABLE IV

COMPARISON OF THE REPEATABILITY OF THE ION-PAIR METHOD WITH THE OFFICIAL METHOD

Method	*Average,* $\bar{x}_{10}$ *(μg/g)*	*Standard deviation,* S_{rel} *(%)*
Ion-pair	92.6	1.5
Official	92.7	4.1

TABLE V

4-MeI CONTENTS OF VARIOUS CARAMEL COLOURS DETERMINED BY THE ION-PAIR METHOD AND BY THE OFFICIAL METHOD

Caramel colour no.	*4-MeI (ppm per 20,000 EBC units)*	
	Ion-pair method	*Official method*
1	351	310
2	143	119
3	82	83
4	170	170
5	6.6	
6	10.1	
7	139	136
8	66	62
9	123	109
10	341	295
11	272	246
12	145	125
13	149	121
14	62	56

Analyses

The content of 4-MeI in various caramel colours was determined by duplicate determinations by both the ion-pair method and the official method. The results are presented in Table V and are expressed by reference to a standard colour intensity of 20.000 EBC units, as recommended by the European Brewery Convention.

The results of both methods were tested for homogeneity of variance at the 5% level with Bartlett's test according to Youden[19], using the relative standard deviations from duplicate determinations. The test confirms that homogeneity of variance exists for both methods.

Comparison of the results with the confidence intervals determined on the basis of the pooled relative standard deviations from each method shows that, at the 99% level and at contents of 4-MeI higher than 300 μg/g, the ion-pair method gives results significantly greater than those of the official method. Moreover, from the recovery results in Table III, it appears that at high levels of 4-MeI the ion-pair method has a better accuracy than the official method.

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REFERENCES

1 *WHO Food Additives Series*, Vol. 7, World Health Organization, Geneva, 1976, p. 35.
2 R. A. Wilks, Jr., A. J. Shingler, L. S. Thurman and J. S. Warner, *J. Chromatogr.*, 87 (1973) 411.
3 R. A. Wilks, M. W. Johnson and A. J. Shingler, *J. Agr. Food Chem.*, 25 (1977) 605.
4 M. Komoto, *Proc. Res. Soc. Japan Sugar Refin. Technol.*, 24 (1973) 51.
5 J. Carnevale, *Food Technol. Aust.*, 27 (1975) 165.
6 G. K. Buckee and T. P. Bailey, *J. Inst. Brew.*, 84 (1978) 158.
7 G. Fuchs and S. Sundell, *J. Agr. Food Chem.*, 23 (1975) 120.
8 M. Cerny and A. Blumenthal, *Z. Lebensm.-Unters.-Forsch.*, 168 (1979) 87.
9 W. A. Davis and C. G. Hartford, in George Charalambous (Editor), *Liquid Chromatographic Analysis of Food and Beverages*, Vol. 2, Academic Press, New York, 1979, p. 353.
10 D. Willumsen, *Report No. 79013*, National Food Institute, Denmark.
11 J. White and D. J. Munns, *J. Inst. Brew.*, 53 (1947) 305.
12 *FAO Nutrition Meetings Report Series*, Vol. 57, Food and Agriculture Organization of the United Nations, World Health Organization, Rome, 1977, p. 39.
13 P. Helboe and M. Thomsen, *Arch. Pharm. Chem. Sci. Ed.*, 5 (1977) 25.
14 R. Modin and M. Johansson, *Acta Pharm. Suecica*, 8 (1971) 561.
15 F. Schneider, *Hoppe-Seyler's Z. Physiol. Chem.*, 338 (1964) 131.
16 R. Modin, *Acta Pharm. Suecica*, 8 (1971) 509.
17 R. Modin and G. Schill, *Talanta*, 22 (1975) 1017.
18 P. Helboe and M. Thomsen, *Int. J. Pharm.*, 2 (1979) 317.
19 W. J. Youden, *Statistical Methods for Chemists*, Wiley, New York, 1951, p. 21.