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# Gas chromatographic–mass spectrometric determination of 4-(5) methylimidazole in ammonia caramel colour using ion-pair extraction and derivatization with isobutylchloroformate

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**Abstract**

A procedure for the gas chromatographic–mass spectrometric quantification of 4-(5-)methylimidazole in ammonia caramel colours by using isobutylchloroformate as derivatizing reagent has been developed. The use of this reagent coupled to a previous ion-pair extraction of the compound with Bis-2-ethylhexylphosphate enabled routine determination of 4-(5) methylimidazole with several advantages compared to others methods previously described. Examples of the analysis of some samples are presented as well as data on linearity, recovery and repeatability. Under the adopted conditions the limit of detection and the limit of quantification were 0.250 mg $kg^{-1}$ and 1 mg $kg^{-1}$, respectively. © 1997 Elsevier Science B.V.

*Keywords:* Derivatization, GC; Ammonia caramel colours; 4-(5-)Methylimidazole; Isobutylchloroformate

## 1. Introduction

4-(5-)Methylimidazole [4-(5-)MI] is a nitrogen heterocycle with a strong convulsive activity, able to cause states of hyperexcitability in several animals such as rabbits, mice and chicks [1] and cattle [2–4]. Moreover, a recent study has demonstrated its capacity, similar to that of pyridine or pyrrole, to inhibit a cytochrome P450 isoenzyme which catalyzes, in the human liver, the oxidation of many known or suspected carcinogens of low-molecular-mass [5].

For humans, the main sources of 4-(5-)MI are the ammonia caramel colours, a group of additives widely used in the food industry to colour a large number of commonly consumed foods and drinks, notably beers, carbonated beverages, cakes and biscuits, meat products, pickles, sauces and confectionery [6]. The presence of 4-(5-)MI in these type of colourings is a result of the reactions which are secondary to those involved in formation of the colouring matter [7]. Regarding its toxicity the World Health Organisation (WHO) has specified, as a precautionary measure, the acceptable limit of 4-(5) MI as 200 mg $kg^{-1}$ based on a caramel colour having a colour intensity of 20 000 EBC units [8].

Considering its chemical properties, especially its strong polar character, the quantification of 4-(5-)MI in complex matrices such as ammonia caramel colours, is a difficult task with respect to both the extraction process and the final determination. Accordingly, the methods which have been proposed for its determination exhibit several important limitations.

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During the seventies a few methods suggesting the use of gas chromatography (GC) were proposed. These consisted, mainly, in a solvent extraction of the sample, directly [9–11] or upon mixture in a column with Celite [12,13] or after a clean-up in an ion-exchange column [6,14] followed by a GC analysis, without derivatization in a column with an alkali treated phase [9–13], or after N-acetylation [6,8,14] as described by Begg and Grimmet [15]. None of these methods were entirely satisfactory when accuracy, easy of use and analysis time were considered. The extraction procedures were fastidious, very time-consuming and showed low and irreproducible yields. On the other hand, GC analysis also posed several drawbacks. Although the analysis of the underivatized compound leads to serious problems of adsorption and consequent tailing, the proposed acetylating reaction for derivatization of the compound is of difficult standardization due to the high sensitivity towards moisture and short period of stability of the acetyl derivative of 4-(5) MI.

In 1981, Thomsen and Willumsen [16] developed a method consisting of an ion-pair extraction with bis-2-ethylhexylphosphate (BEHPA) in chloroform and subsequent analysis of the resulting extract by ion-pair high-performance liquid chromatography (HPLC) with UV detection at 215 nm. Notwithstanding the poor detection limit obtained (5 mg $kg^{-1}$) and the difficulties associated with the use of ion-pair HPLC techniques, this method proved to be a great improvement when compared with the existing methods, especially with regards to the extraction process. As a result it was followed thereafter, with minor modifications, by several authors in the determination of the compound not only in ammonia caramel colours but also in other matrices such as plasma [17,18] and animal urine [17–19], milk [17,18] and fodder [18–20]. More recently, proposals have been made for the determination of 4-(5-)MI using thin-layer chromatography (TLC) [21–23], fluorimetry [24], capillary isotachophoresis [25] and capillary electrophoresis (CE) [26], and also by a process of online coupling of HPLC, solid-phase extraction (SPE) and TLC [27].

This paper describes a new and useful GC method for the quantification of 4-(5-)MI in ammonia caramel colours in which the compound is derivatized with isobutylchloroformate (IBCF) after an ion-pair extraction with BEHPA in chloroform. The advantages of this method comparing with others previously developed are considered.

## 2. Experimental

### 2.1. Chemicals

4-(5-)MI was obtained from Sigma (St. Louis, MO, USA). Imidazole and 2-methylimidazole were obtained from Aldrich (Steinheim, Germany). 2-Ethylimidazole was obtained from Aldrich (Milwaukee, WI, USA). Stock standard solutions of each imidazole at 2.0 mg $ml^{-1}$ were prepared by weighing and dissolving them in 0.1 *M* HCl and stored at 4°C in silanized screw capped vials with solid PTFE lined caps (Supelco, Bellefont, PA, USA). Working standard solutions were prepared by dilution and mixing of these solutions with 0.1 *M* HCl and stored in the same way as the stock standard solutions.

BEHPA was obtained from Aldrich and IBCF was obtained from Sigma. Chloroform (SupraSolv quality) and acetonitrile (LiChrosolv quality) were obtained from Merck (Darmstadt, Germany). Pyridine (over molecular sieve, purity>99.8%) and isobutanol (purity>99.8%) were obtained from Fluka (Buchs, Switzerland). All the other reagents were analytical grade. Water was prepared by purifying demineralized water in a Seral system (Seralpur Pro 90 CN).

### 2.2. Ion-pair extraction

The calibrant and sample solutions were similarly prepared by weighing 3.00 g of caramel colour [non-ammoniacal caramel colour free of 4-(5-)MI with respect to the calibrant solutions] into a 25 ml volume flask with subsequent dilution using approximately 15 ml of 0.2 *M* potassium phosphate buffer pH 6. The internal standard dissolved in phosphate buffer was added to both calibrant and sample solutions, whereas different quantities of 4-(5-)MI were added to the calibrant solutions alone. After thorough mixing of the contents, the pH was adjusted to 6.0 by dropwise addition of concentrated potassium hydroxide solution. Finally, phosphate

buffer was added to fill the measuring cylinder to the 25 ml mark.

A 1-ml aliquot was transferred to a silanized screw capped vial and extracted with 2 ml of 0.1 *M* BEHPA in chloroform, by thorough hand mixing for 2 min and subsequent stirring for 1 min on a vortex-mixer. After separation of the phases by centrifuging at 3500 *g* for 10 min, a 1.5 ml portion of the chloroform phase (bottom layer) was further removed to a second vial which contained 1.5 ml of 0.1 *M* HCl and by shaking as previously described, the amount of 4-(5-)MI and internal standard were back-extracted into the aqueous phase. Upon separation by centrifugation the resulting aqueous phase (upper layer) was ready for derivatization and subsequent chromatographic analysis.

### 2.3. Derivatization

A 250-μl aliquot of the aqueous phase obtained via the extraction procedure (or the same volume of a working standard solution) were added with a 250-μl aliquot of the acetonitrile–isobutanol–pyridine (50:30:20, v/v) mixture in a silanized screw capped vial. Subsequently 25 μl of IBCF were added. After a brief shaking (4–5 s), 500 μl of a 1.0 *M* aqueous natrium bicarbonate solution and 500 μl of chloroform were added and the mixture was mixed for a further 4–5 s resulting in a chloroform phase (bottom layer) ready for chromatographic analysis.

### 2.4. Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out using a HP 5890 chromatograph equipped with a split/splitless capillary inlet system and interfaced to a HP MSD-5970 B mass selective detector (70 eV, electron impact mode) controlled by a HP Chemstation. GC separation was achieved on a DB-5 MS capillary column (J&W Scientific, Folsom, CA, USA) 30 m×0.25 mm I.D., film thickness 0.25 μm, coupled directly to the mass detector. A persilanized (2 m×0.25 mm) fused-silica piece (retention-gap) was introduced between the DB-5 MS column and the injector. Periodically a 20-cm piece was cut-off.

A 1.2-μl aliquot of the extract was injected in the splitless mode at 250°C. The purge-off time was 1.0 min. The initial column temperature was 70°C, maintained for 1.0 min and then programmed at 20°C $min^{-1}$ to 280°C. The temperature of the transfer line was 280°C. Helium N 60 (purity of 99.9999%) was employed as carrier gas with a head pressure of 80 KPa.

The detector was optimized under auto-tuning conditions with perfluorotributylamine. The acquisition was performed in the full-scan mode during the optimization of the chromatographic method as well as for the obtention of the spectra for each analyzed imidazole derivative. Quantification of 4-(5-)MI was performed in the selected ion-monitoring mode (SIM). Ions *m/z* 68, 81, 82, 95, 109, 168 and 182 were used with a "dwell time" of 30 ms each. The detector was switched off in the initial 5 min of each chromatographic run in order to ensure the removal of the solvent and the excess of IBCF without damaging the filament.

## 3. Results and discussion

### 3.1. Ion-pair extraction

BEHPA is an ion-pairing and adduct forming agent used in the extraction of basic organic compounds [28–31], which was successfully applied by Thomsen and Willumsen in the extraction of 4-(5)MI from ammonia caramel colours [16]. The procedure reported herein represents a slight improvement toward the methodology previously described by these authors. Modifications included (i) the change of the volume ratio of caramel solution–BEHPA solution from 1:1 to 1:2 in order to allow for a better separation of the two phases, which otherwise was incomplete in some of the caramel samples; (ii) the use of smaller extraction volumes (1 ml+2 ml instead of 4 ml+4 ml); (iii) the use of 0.1 *M* HCl instead of 0.1 *M* $H_3PO_4$ in the back-extraction step to prevent the turbidity observed in the final organic phase (chloroformic extract) obtained upon derivatization, when $H_3PO_4$ was used.

The present process proved to be very effective for the extraction of all imidazole derivatives assayed.

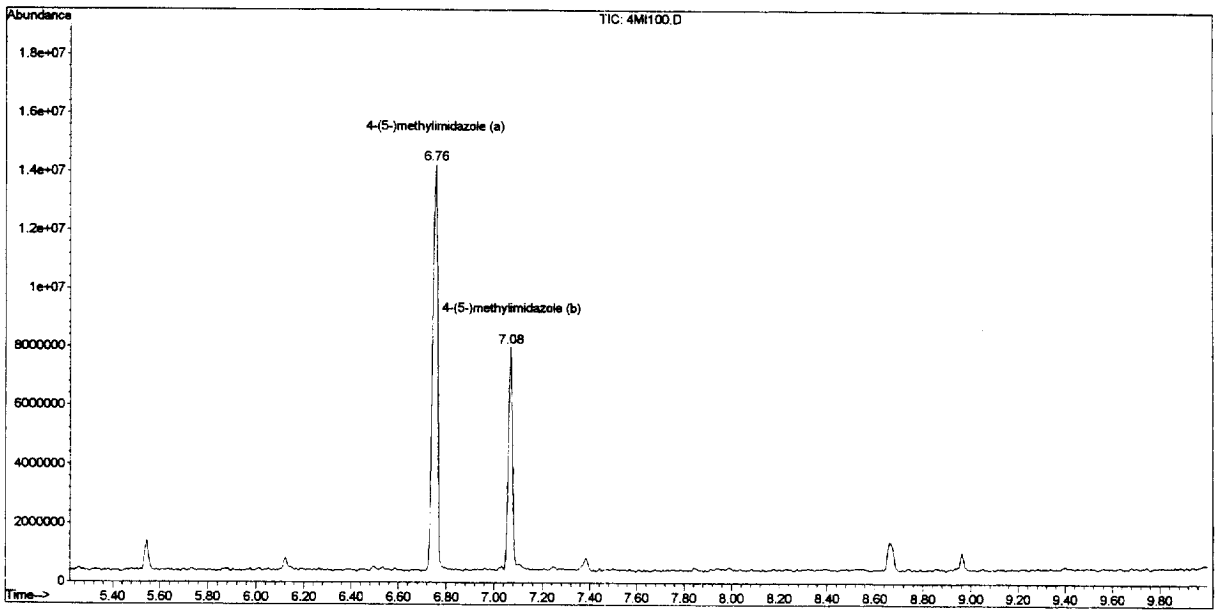


Fig. 1. Total ion chromatogram (full-scan 40–450 u) of a pure standard solution (100 mg $l^{-1}$) of 4-(5-)MI derivatized with isobutylchloroformate (conditions given in Section 2).

### 3.2. Derivatization

The direct GC of 4-(5-)MI is not recommended, as with amine compounds in general, since its high polarity and hydrogen-bonding tendency makes the appearance of a symmetrical peak impossible, a more or less pronounced tailing is always noted [15,32].

As mentioned above an updated literature review has shown acetylation to be the only process referred for the derivatization of 4-(5-)MI for posterior GC analysis. However, the process has certain limitations, which include, the high sensitivity of the reaction to water present in the apparatus or reagents and the unstable nature of the acetylated 4-(5-)MI molecule which urges a time standardization requirement between derivatization and chromatography [14]. In fact, the high reactivity of acetylimidazoles, in general, (half-life of acetylimidazole in water at 25°C and pH 7 is 41 min) makes them suitable as acetylating reagents of acid-sensitive compounds [33].

During the development of a more appropriate method for GC analysis of 4-(5-)MI in caramels, the alkylchloroformates (CFs) were selected for their good performance. This group of reagents is especially useful for the derivatization and subsequent GC analysis of amines resulting in the formation of the respective N-alkoxycarbonil esters with good GC properties. Originally, this reaction was promoted in an alkaline medium, during 10 min at room temperature [34,35]. More recently, Husek, reported that under certain conditions, the CFs were able to react with amine groups (as well as other groups including acids) in a practically instantaneous and quantitative manner. Such conditions implied the presence of pyridine, acetonitrile and the alcohol corresponding to the alkyl group of the employed chloroformate, in variable proportions according to the type of compound to be derivatized [36,37].

Taking into account the above mentioned considerations, IBCF was chosen for further derivatization experiments of 4-(5-)MI. Results from the different trials performed using different relative concentrations of the various reagents involved, concluded that under the adopted environmental conditions – 0.1 *M* aqueous hydrochloric acid (containing the 4-(5-)MI to be analyzed)–acetonitrile–isobutanol–pyridine (50:25:15:10, v/v) – the addition of an amount of IBCF half that of the pyridine used, originated an

immediate esterification of practically all the available 4-(5-)MI. The reaction lead to the formation of two different compounds (see further on) which were easily extracted by chloroform once 1.0 *M* sodium bicarbonate solution was added.

The developed derivatization procedure is quite adequate for the proposed aim, since it reveals certain positive aspects worthy of mention. The chemical conversion occurs rapidly (4–5 s) proceeding at room temperature and in the presence of dilute hydrochloric acid which allows for a close link with the extraction procedure employed. Furthermore, there is no need for an evaporation step of the solvent, thus preventing a decrease of the accuracy and precision which normally occurs under such circumstances.

All the imidazole derivatives were stable and no decomposition was observed even after standing in the vial, in which the derivatization and subsequent chloroform extraction had occurred, for 4 weeks (at least) at room temperature. This fact is an extremely important advantage in relation to methods based on the acetylation of the compound.

### *3.3. Gas chromatography–mass spectrometry*

As shown in Fig. 1 the GC–MS analysis of the 4-(5-)MI derivatized with IBCF, revealed the presence of two well-defined and totally separated peaks – 4-(5-)MI (a) and 4-(5-)MI (b) – and these appeared systematically in both pure standards and caramel samples. Both peaks show quasi-identical spectra (Fig. 2) with a characteristic base peak at *m/z* 41 the aziridine cation which results from the elision of $CH_3CN$ from the imidazole ring [38] and a molecular ion peak ($M^+$) at *m/z* 182 corresponding to the parent compound. Other common peaks were *m/z* 57, a non-specific fragment common to all isobutoxycarbonyl derivatives, *m/z* 82 and *m/z* 81, corresponding to the 4-(5-)MI molecule with or without a proton on one of the nitrogens of the imidazole ring, respectively, and *m/z* 109, $M^+-73$ $[OCH_2CH(CH_3)_2]$. Besides the insignificant differences in relation to the relative intensity of the common fragments, the only important difference between the two spectra is the presence of a fragment *m/z* 68 in the derivative with less retention

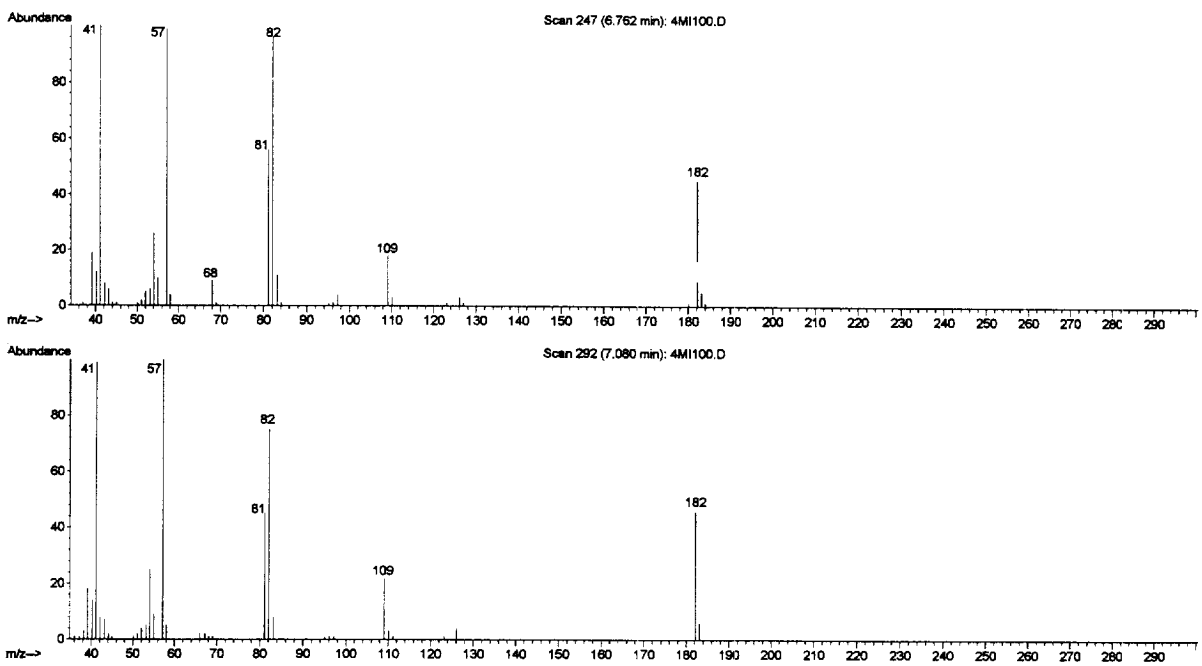


Fig. 2. EI mass spectra of the two 4-(5-)MI derivatives resulting from the derivatization of the compound with isobutylchloroformate [4-(5-)MI (a), top; 4-(5-)MI (b), bottom].

time, which corresponds to the imidazole group and which is absent in the second derivative.

The presence of these two peaks is probably due to the existence of tautomerism equilibria in the 4-(5-)MI molecules a well-known phenomenon which consists in a proton chemical shift between the two nitrogens of the imidazole ring [39,40]. To our present knowledge, although experimental studies are available on the separation of each tautomer based on the formation of N-methylated derivatives of 4-(5-)MI [40], our study is the first to refer the separation of two acylated derivatives of 4-(5-)MI. Generally, acylation of 4-(5-)MI originate a single derivative, viz. the one with no steric hindrance [32,41].

The relative proportion between the two peaks obtained from derivatization of 4-(5-)MI and calculated based on the respective areas, showed some variation even when consecutive injections of the same standard or sample were considered. The first peak was always higher than the second which, in turn, exhibited an area about 30–45% of the former. This was, however, no constraint for a correct quantification of the compound, since the sum of the peak areas was used.

As internal standard, three other imidazoles, viz. imidazole, 2-methylimidazole and 2-ethylimidazole, absent in any of the caramels analyzed, were assayed for. All three compounds showed a good behavior in both the ion-pair extraction process and the GC analysis, registering a single well-defined peak with a symmetrical shape (Fig. 3). These results lead us to believe that the developed methodology could be used with great advantage in studies of simultaneous quantification of different imidazoles. When used as an internal standard, 2-methylimidazole had the disadvantage of registering a retention time very close to that of the 4-(5-)MI (a) which, coupled to the similarity of the respective spectra, prevented the correct quantification of the areas of the two peaks obtained with the temperature programme used. (The total separation of the two compounds was, however, possible with a slower rise in the temperature programme or in a column with higher polarity – DB 35 MS). Similar characteristics were reported by imidazole and 2-ethylimidazole, and although either compound could be used as an internal standard, our final choice was for imidazole.

The use of a mass spectrometer as a detector allowed for the application of a "rapid" temperature

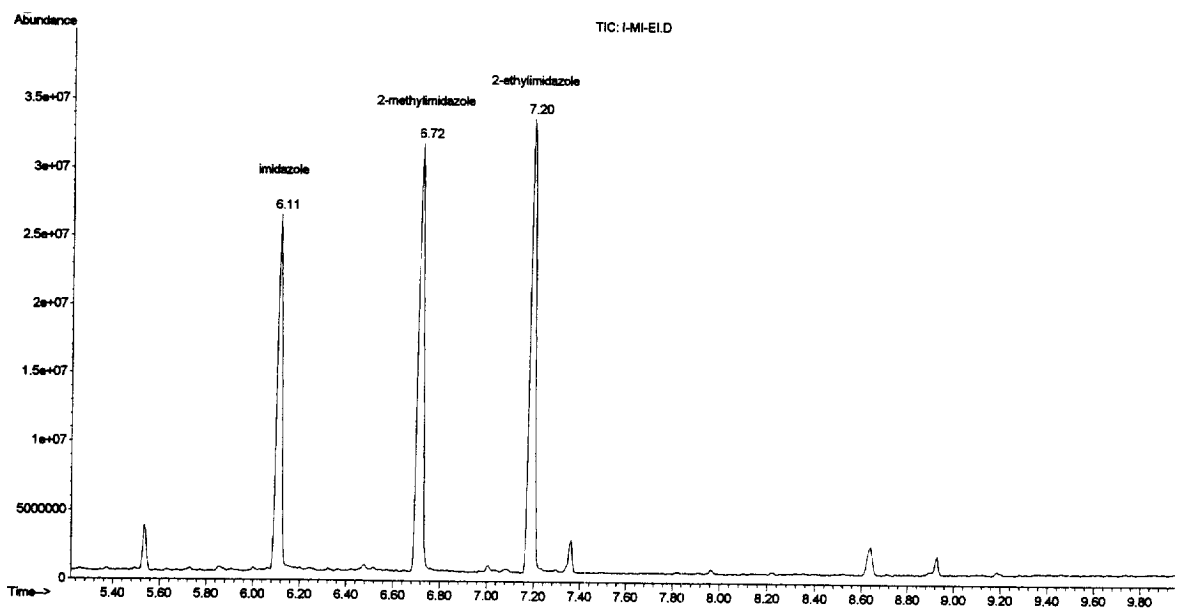


Fig. 3. Total ion chromatogram (full-scan 40–450 u) of a standard solution of imidazole, 2-methylimidazole and 2-ethylimidazole (100 mg $l^{-1}$ each) derivatized with isobutylchloroformate (conditions given in Section 2).

programme and the consequent gain in analysis time, otherwise impracticable. In fact, under the conditions used, the separation of the peaks corresponding to the compounds of interest, especially 4-(5-)MI (b), from those of interfering compounds present in some samples was not complete. Given their specificity, the use of reconstructed chromatograms of the ions *m/z* 182, with regard to 4-(5-)MI (a) and 4-(5-)MI (b), and *m/z* 168 with regard to imidazole did, however, permit the obtention of perfectly separated peaks and the easy integration of the respective areas (Fig. 4). The use of another detector e.g., flame ionization detector (FID) is, in our opinion, also perfectly possible, although due to the facts already mentioned it implies an increase in the analysis time. Further laboratory work needs to be carried out in order to fully support our suggestion.

The column which was employed – DB 5 MS – proved to be perfectly adequate for the analysis and no visible degradation was noted during the whole length of the experimental work. Our experience also noted that the use of a pre-column improved the quality of the obtained chromatograms.

### *3.4. Linearity, recovery and repeatability*

The linearity of the overall method was tested using "real" calibrant solutions consisting of five samples of the same non-ammoniacal caramel colour [previously tested for the absence of any small quantity of 4-(5-)MI and imidazole] added with known amounts of 4-(5-)MI corresponding to 5, 25, 100 and 250 mg $kg^{-1}$ and a known amount of the internal standard (200 mg $kg^{-1}$). The resulting calibrant solutions were extracted and analyzed according to the described procedures. Each final chloroformic extract was injected twice and a calibration curve was constructed by means of relative response factors. The results obtained demonstrate an excellent linearity with a correlation coefficient of 0.9998, a small intercept of $0.001848 \pm 0.003249$ and a slope of $0.003703 \pm 0.000027$.

The reliability of the method was confirmed by a recovery experiment. Samples of an ammonia caramel colour with an 4-(5-)MI amount of 41.8 mg $kg^{-1}$ were spiked before extraction with 20.0, 40.0, 80.0 and 160 mg $kg^{-1}$ of 4-(5-)MI. Upon addition of

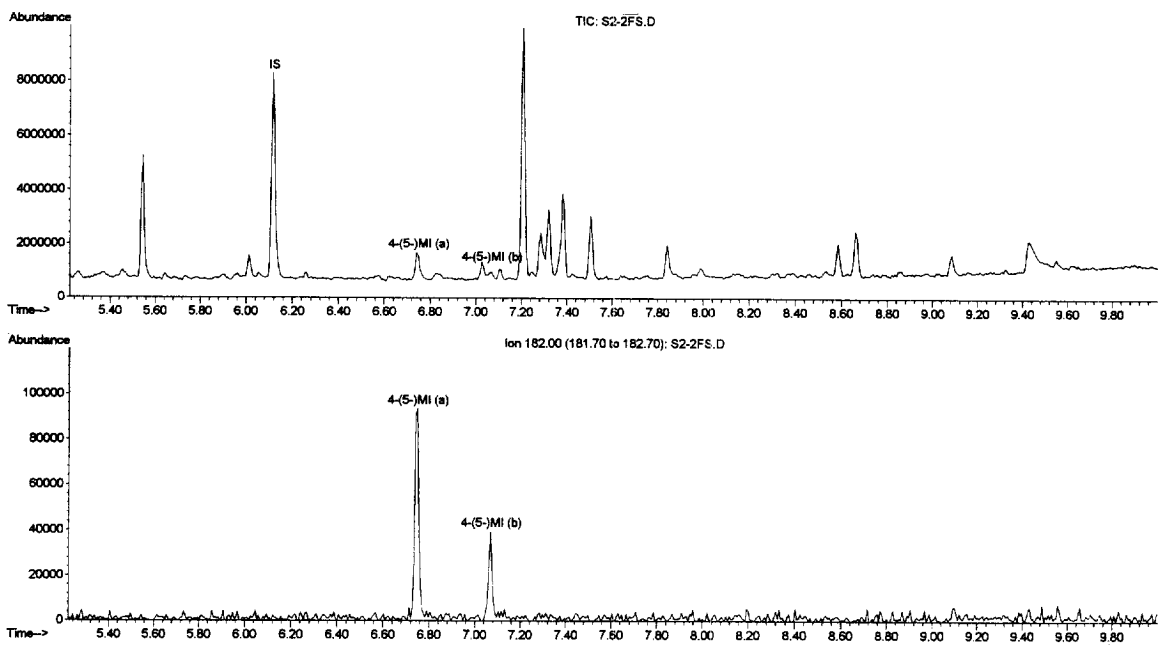


Fig. 4. Total ion chromatogram (full-scan 40–450 u) (top) and corresponding reconstructed ion chromatogram *m/z* 182 (bottom) obtained from a sample of ammonia caramel colour with 41.8 mg $kg^{-1}$ of 4-(5-)MI submitted to the developed analytical methodology.

Table 1
Mean recoveries of 4-(5-)MI from spiked ammonia caramel samples

| 4-(5-)MI (mg kg$^{-1}$) | | | Recovery (%) |
|---|---|---|---|
| Initial amount | Added | Found[a] | |
| 41.8 | 20 | 60.80 | 95.0 |
| 41.8 | 40 | 82.60 | 102.0 |
| 41.8 | 80 | 120.30 | 98.1 |
| 41.8 | 160 | 203.05 | 100.8 |

[a] Mean value of two injections.

Table 2
Repeatability data for two samples of ammonia caramel colour with different amounts of 4-(5-)MI ($n=10$)

| Sample | Mean (mg kg$^{-1}$) | S.D. | R.S.D. (%) |
|---|---|---|---|
| A | 211.96 | 2.913 | 1.4 |
| B | 41.79 | 0.769 | 1.8 |

the internal standard, the samples were subject to the analytical procedure previously described and each extract was injected twice. The results obtained are shown in Table 1.

The repeatability of the overall method was tested in two samples of ammonia caramel containing different concentrations of 4-(5-)MI (41.8 and 212.0 mg kg$^{-1}$). In both cases, 10 aliquots of the same sample were prepared and added with internal standard, after which extraction, derivatization and GC analysis were duly performed. Results are presented in Table 2.

### 3.5. Limit of detection and limit of quantification

The limit of detection of the method was estimated at 0.250 mg kg$^{-1}$ of 4-(5-)MI at a signal-to-noise ratio of 5 for the 4-(5-)MI (a), corresponding to 0.005 ng μl$^{-1}$ in the injected extract. The limit of quantification was somewhat higher, approximately

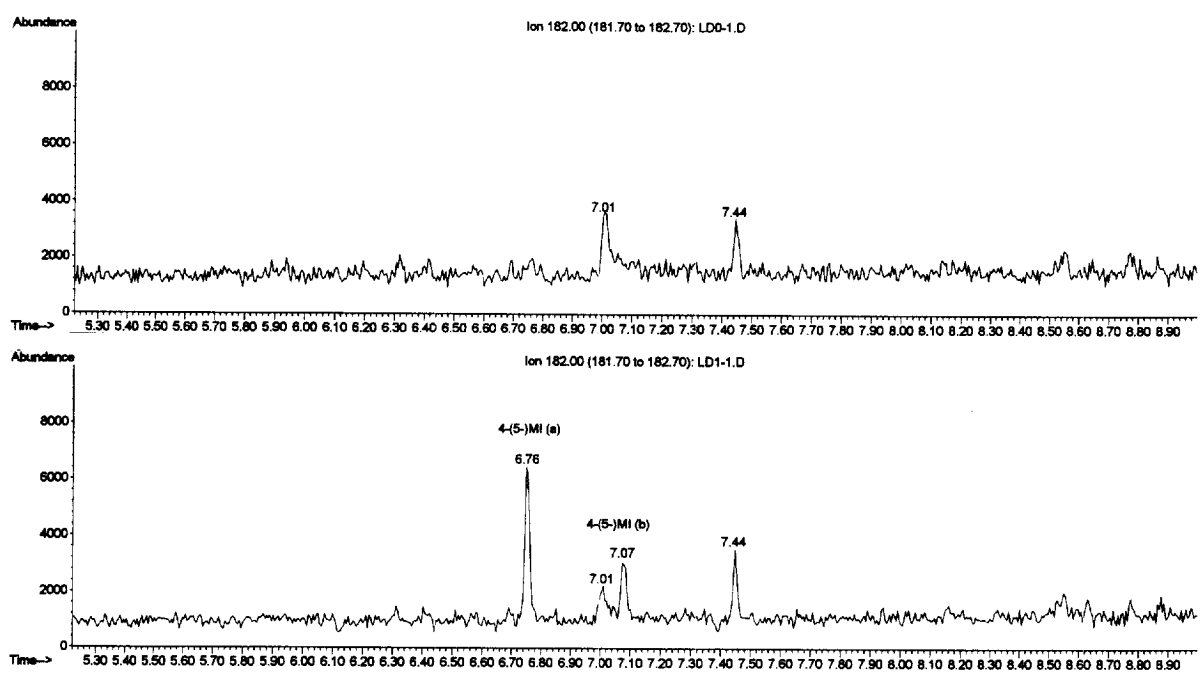


Fig. 5. Reconstructed ion chromatograms $m/z$ 182 obtained under the conditions used for quantification (selected ion monitoring of seven ions) from a blank caramel sample free of 4-(5-)MI (top) and from the same sample added with 1.0 mg kg$^{-1}$ of 4-(5-)MI, corresponding to the estimated limit of quantification of the developed method (bottom).

Table 3
4-(5-)MI contents of some ammonia caramel colours and malt extract samples determined by the developed method

| Samples | | Amount (mg $kg^{-1}$) |
|---|---|---|
| Ammonia caramel colour | A | 212.0 |
| | B | 41.8 |
| | C | 103.8 |
| | D | 7.5 |
| Malt extract | E | 0 |
| | F | 0 |

1.0 mg $kg^{-1}$ (Fig. 5). Since the quantification was made with the sum of the peak areas of 4-(5-)MI (a) and 4-(5-)MI (b), the size of the latter was, in this case, the limiting factor.

### 3.6. Results

The developed methodology was applied in the quantification of the 4-(5-)MI level in four ammonia caramel colour and two malt extract samples with different origins. Results obtained are presented in Table 3.

## 4. Conclusions

The method described is suitable for the quantification of the 4-(5-)MI in ammonia caramel colours. The previously reported difficulties encountered when acetylation was used for the GC analysis of 4-(5-)MI were solved by using IBCF as a derivatising reagent. Under the adopted conditions, the overall method, including extraction, derivatization and GC analysis, may be performed in less than an hour, which represents a large gain in analysis time in relation to the existing GC methods. Linearity and repeatability are quite good in a broad range of concentrations and the limit of detection is low enough to verify the presence of small quantities of the compound. In addition, the method allows for the determination of 4-(5-)MI as well as other possibly existing imidazole derivatives. The application of this method to other matrices, namely, food products containing very low levels of 4-(5-)MI, appears to be quite possible and efforts are being carried out to confirm such viability.

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## References

[1] K. Nishie, A.C. Waiss Jr., A.C. Keyl, Toxicol. Appl. Pharmacol. 14 (1969) 301.
[2] S.E. Morgan, W.C. Edwards, Vet. Hum. Toxicol. 28 (1986) 16.
[3] T.K. Nielsen, C. Wolstrup, N. Witt, V.F. Kristensen and I. Kraul, in Proceedings of the 6th International Conference on Production. Diseases in Farm Animals, Belfast, 1986, p. 293.
[4] T.E. Fairbrother, L.A. Kerr, H.W. Essig, Vet. Hum. Toxicol. 29 (1987) 312.
[5] M.B. Hargreaves, B.C. Jones, D.A. Smith, A. Gescher, Drug Metab. Dispos. 22 (1994) 806.
[6] A.L. Patey, G. Shearer, M.E. Knowles, W.H.B. Denner, Food Addit. Contam. 2 (1985) 107.
[7] J. Carnevale, Food Technol. Aust. 27 (1975) 165.
[8] WHO Food Additives Series, Vol. 7, World Health Organisation, Geneva, 1976, p. 35.
[9] G. Fuchs, S. Sundell, J. Agric. Food Chem. 23 (1975) 120.
[10] M. Cerny, A. Blumenthal, Z. Lebensm-Unters.-Forsch. 168 (1979) 87.
[11] L. Dagna, M. Fenocchio, G. Gasparini, Bollett. Chimici Lab. Provinic. 6 (1980) 327.
[12] R.A. Wilks, A.J. Shingler, L.S. Thurman, J.S. Warner, J. Chromatogr. 87 (1973) 411.
[13] R.A. Wilks, M.W. Johnson, A.J. Shingler, J. Agric. Food Chem. 25 (1977) 605.
[14] G.K. Buckee, T.P. Bailey, J. Inst. Brew. 84 (1978) 158.
[15] C.G. Begg, M.R. Grimmett, J. Chromatogr. 73 (1972) 238.
[16] M. Thomsen, D. Willumsen, J. Chromatogr. 211 (1981) 213.
[17] P. Nielsen, C. Friis, I. Kraul, Res. Vet. Sci. 54 (1993) 72.
[18] T. Sivertsen, K. Ingebrigsten, Acta Vet. Scand. 34 (1993) 227.
[19] E. Karangwa, G.E. Mitchell Jr., R.E. Tucker, J. Chromatogr. 532 (1990) 105.
[20] J. Bergström, J. Agric. Food Chem. 39 (1991) 1422.
[21] L. Magrian, J. Pagenkopf, M. Schilling, U. Sell, Dtsch. Lebensm. Rundsch. 81 (1985) 379.
[22] G. Lehmann, B. Binkle, Lebensmittelchem. Gerichtl. Chem. 41 (1987) 9.
[23] E. Rabe, W. Seibel, K. Wuenscher, Mehl. Brot. Getreide 42 (1988) 142.
[24] M.C. Gutierrez, A. Gomez-Hens, M. Valcarcel, J. Microchem. 34 (1986) 332.
[25] F. Kvasnicka, Electrophoresis 10 (1989) 801.

[26] C.P. Ong, C.L. Ng, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 686 (1994) 319.
[27] E. Mueller, H. Jork, J. Planar Chromatogr. Mod. TLC 6 (1993) 21.
[28] R. Modin, G. Schill, Talanta 22 (1975) 1017.
[29] R. Modin, M. Johansson, Acta Pharm. Suecica 8 (1971) 561.
[30] G. Hoogewijs, D.L. Massart, Anal. Chim. Acta 106 (1979) 271.
[31] G. Hoogewijs, D.L. Massart, J. Pharm. Biomed. Anal. 2 (1984) 449.
[32] P. Moree-Testa, Y. Saint-Jalm, A. Testa, J. Chromatogr. 290 (1984) 263.
[33] K. Blau, in K. Blau and J. Halket (Editors), Handbook of Derivatives for Chromatography, Wiley, Chichester, 1994, Ch. 3, p. 33.
[34] M. Makita, S. Yamamoto, M. Miyake, K. Masamoto, J. Chromatogr. 156 (1978) 340.
[35] O. Gyllenhaal, L. Johansson, J. Vessman, J. Chromatogr. 190 (1980) 347.
[36] P. Husek, LC·GC Int 9 (1992) 43.
[37] P. Husek, Anal. Chim. Acta 259 (1992) 185.
[38] Q.N. Porter and J. Baldas, in A. Weissberger and E.C. Taylor (Editors), Mass Spectrometry of Heterocyclic Compounds, Wiley Interscience, New York, 1971, Ch. 12. p. 451.
[39] M.R. Grimmett, in P.G. Sammes (Editor), Comprehensive Organic Chemistry—The Synthesis and Reactions of Organic Compounds, Vol. 4—Heterocyclic Compounds, Pergamon Press, Oxford, Ch. 17, p. 366.
[40] G.A. Worth, P.M. King, W.G. Richards, Biochim. Biophys. Acta 993 (1989) 134.
[41] R.A. Olofson, R.V. Kendall, J. Org. Chem. 35 (1970) 2246.