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Gas chromatographic–mass spectrometric quantification of 4-(5-)-methylimidazole in roasted coffee after ion-pair extraction

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Abstract

A GC–MS method is described for quantification of 4-(5-)methylimidazole (4MI) in coffee. Although tested, GC–flame ionization detection proved inadequate for this purpose due to the complexity of the coffee matrix. The developed method was based on ion-pair extraction with bis-2-ethylhexylphosphate and derivatization with isobutylchloroformate. Quantification was carried out by the standard addition method using 2-ethylimidazole as internal standard. Reproducibility data from the complete procedure are presented. Mean recoveries were higher than 98%. The method was applied to green and roasted coffee samples from the two most important varieties, arabica and robusta, and to commercial ''torrefacto'' coffee blends. 4MI was not detected in the green coffee samples analysed and ranged from 0.307 to 1.241 mg/kg in roasted samples.

Keywords: Coffee; Food analysis; Derivatization, GC; Chloroformates; Methylimidazole; Imidazoles

1. Introduction

4-(5-)Methylimidazole (4MI) is a simple nitrogen-containing heterocyclic compound that may be formed from sugars' reaction with ammonia under specific conditions. It is a central nervous system (CNS) agent that could illicit convulsions in animals and has attracted special attention due to its possible toxicity. The reported oral LD_{50} values for mouse and chicken are 370 and 590 mg/kg, respectively. Because of its wide presence and lack of carcinogenic information, the chronic toxicity of 4MI is currently being evaluated in ''in vivo'' animal studies [1]. Meanwhile, the World Health Organisation has recommended a limit content of 200 mg/kg for ammonia process caramels, one of the major known sources of this compound for humans.

Accurate methods to determine 4MI content in several products, namely food, are needed, in order to determine the ingested amounts. This compound has been detected in several beverages coloured with caramel, like beer, colas or fruit juices, and other foods such as milk, sauces, roasted barley and bread. Presently there is no information available about the possible concentration of 4MI in coffee. As ammonia content in coffee is negligible [2] it is not expected to find high levels of 4MI from the reaction of sugars with ammonia, the more traditional reported source. However it is known that 4MI can also be formed by pyrolysis, such as in cigarette smoke [3]. Since coffee is submitted to a roasting process, more or less intense according to requirements, we were aiming to verify the possible presence of 4MI in roasted coffee.

The coffee matrix is an extremely complex one,

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since several thousand different compounds are present, especially after roasting. An efficient clean-up procedure is therefore necessary in order to achieve correct quantification of 4MI. Based on reported clean-up methods, ion-pair extraction seemed the most selective one and was therefore chosen for this work. The possibility to determine 4MI in coffee by gas chromatography using both flame ionisation detection (GC–FID) and mass spectrometry (GC–MS) is discussed in detail.

2. Experimental

2.1. Chemicals

4-(5-)Methylimidazole was from Sigma (St Louis, MO, USA). The internal standards imidazole (I) and 2-ethylimidazole (EI) were from Aldrich (Steinheim, Germany). Stock standard solutions at 1.0 mg/ml were prepared in 0.1 *M* HCl and stored at 4 °C. Working solutions were prepared by dilution with 0.1 *M* HCl and stored under the same conditions. Ethylchloroformate (ECF), isobutylchloroformate (IBCF), ethanol, isobutanol and pyridine were from Fluka (Neu-Ulm, Germany) and bis-2-ethylhexylphosphate (BEHPA) was from Aldrich. All other chemicals were analytical grade or higher.

2.2. Coffee samples

A *Coffea arabica* sample from Brazil and a *C. robusta* from Ivory Coast were analysed, either green or after a standard industrial roast procedure (160–220 °C, 14 min). Commercial blends of ''torrefacto coffee'' (coffee roasted in the presence of sugar) with ''natural coffee'' were also analysed.

All samples were ground to pass a 25-mesh sieve. Loss in mass at 105 °C was determined according to ISO 11294-1994, immediately after grinding and weighing for extraction, in order to express the results on a dry weight basis.

2.3. Extraction

A 2.00 g portion of each powdered coffee was extracted three times with methanol, after addition of the internal standard (EI). The methanolic extract was dried under nitrogen, at ambient temperature, and redissolved in 4 ml of phosphate buffer (0.2 *M*; pH 6). The tube was capped and vortex-mixed for 30 s. After centrifugation at 3500 *g*, 3 ml were removed to a second tube and extracted with an equal amount of 0.1 *M* BEHPA in chloroform and again vortex-mixed to extract 4MI. After centrifugation, 2 ml of the lower phase (chloroformic) were transferred to a third tube containing 2 ml of 0.1 *M* HCl. Upon shaking and centrifugation, the upper hydrochloric phase was ready for derivatization.

2.4. Derivatization

Derivatization was performed as described by Fernandes et al. [4] with minor modifications. A second internal standard (heptylamine), used to control the derivatization step efficiency, was added to a silanized screw cap tube containing 1000 μl of the aqueous extract. Acetonitrile (150 μl), pyridine (100 μl) and alcohol (300 μl), either ethanol or isobutanol, were added and vortex-mixed. Subsequently, 75 μl chloroformate (ethylchloroformate or isobutylchloroformate) was added. After a brief shaking, 1 ml of a 1.0 *M* $NaHCO_3^-/CO_3^{2-}$ solution and 250 μl of chloroform were added and the mixture further vortex-mixed for 4–5 s for extraction of the derivatives into the organic layer. After centrifugation at 3500 *g* for 1 min, the vials were stored below 4 °C until chromatographic analysis. Then, about 1.2 μl of the chloroformic phase was injected directly into the gas chromatographic system.

2.5. Gas chromatography–flame ionization detection

GC–FID was carried out on a Chrompack CP-9001 gas chromatograph equipped with an automatic Liquid Sampler CP-9050 (Chrompack, Netherlands).

The temperatures of the injector and detector were 250 and 280 °C, respectively. Helium was used as carrier gas at an initial inlet pressure of 80 kPa. Splitless injection was used with a purge time delay of 1 min. Several programmed temperature gradients (from 70 to 280 °C) were used in order to improve

separation of the compounds from other interfering peaks.

2.6. Gas chromatography–mass spectrometry

GC–MS analysis was carried out using two different Hewlett-Packard (Little Falls, DE, USA) systems. Initially with a HP 5890/MSD-5970B, and later with a HP 6890/MSD-5973N system. Both systems were equipped with a capillary column coupled directly to the mass detector. The injection was carried out at 250 °C in the splitless mode. Purge-off time was set at 1 min. Helium was used as carrier gas with a head pressure of 80 kPa. Initial column temperature was 70 °C programmed with a two-step gradient to achieve 280 °C after 16 min (10 min hold). The transfer line temperature was 280 °C. Calibration of the GC–MS system was carried out weekly using the autotune function of the mass selective detector. Electron impact mass spectra were measured at an acceleration energy of 70 eV. Data acquisition was performed in the full-scan mode during the optimisation of the method and in the selected ion-monitoring mode (SIM) for quantification. Characteristic mass fragments (m/z) of the imidazoles after derivatization with isobutylchloroformate were used: 4MI-a (m/z 68, m/z 82, m/z 182), 4MI-b (m/z 82, m/z 182), 2-ethylimidazole (m/z 196). The m/z 160 was also scanned in order to detect heptylamine, the internal standard used to control the derivatization step. Dwell time in the SIM mode was 30 ms, allowing 4.35 cycles per s. The detector was switched off in the initial 6 min in order to completely remove the excess of isobutylchloroformate that could damage the filament. The overall systems were controlled by Hewlett-Packard Chemstations.

2.7. Chromatographic columns

Three analytical columns were used throughout this study: DB-5MS and DB-1701, both 30 m×0.25 mm I.D. (0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA) and SP-Sil-8CB, 25 m×0.25 mm I.D. (0.12 μm film thickness) (Chrompack, Netherlands). A 2-m guard column was used with all columns and periodically a small piece was cut-off.

3. Results and discussion

3.1. 4-(5-)Methylimidazole identification

After some preliminary studies, the presence of 4-(5-)methylimidazole was confirmed in roasted coffee by GC–MS after derivatization with IBCF. In green coffee, no 4MI was observed. The sample extract was obtained with methanol and no internal standard was used in order to confirm their absence.

3.2. Gas chromatography–flame ionisation detection

Once the presence of 4MI in roasted coffee was confirmed, our first attempt was to analyse it by GC–FID. Aware of the difficulty of this method, two types of derivatives were considered. Based on previous analysis developed in our laboratory [4], we have tested the derivatization method described therein, with isobutylchloroformate–isobutanol–pyridine. Another derivatization method was also tested using ethylchloroformate–ethanol–pyridine as also recently reported by us [5]. The reagent quantities were adjusted in order to achieve a more concentrated final extract. Two internal standards (IS) were used: I and EI.

The derivatization methods were examined for linearity of the calibration plots. The peak areas of 4MI and IS were measured and the peak-area ratio calculated. A linear response relationship was obtained with the correlation coefficients being above 0.9999 in the range 2–250 μg/ml with both IS (without IS the correlation coefficient was as small as 0.99). Relative standard deviations (RSDs) at the mid-point of the calibration graphs were lower than 1% from three replicates. Without IS, the same RSD was around 5%. The minimum detectable amounts to give a signal-to-noise ratio of 3 under our GC conditions was 0.1 ng/μl injected.

Fig. 1 represents a chromatogram of standards at 250 mg/l on the DB5-MS column, derivatized with ethylchloroformate. As observed two peaks corresponding to 4MI (a and b) were detected. This observation was valid either for standards or samples and it was already described by Fernandes et al. [4]. It is probably due to the natural tautomerism characteristic of substituted imidazoles [6,7]. Table 1

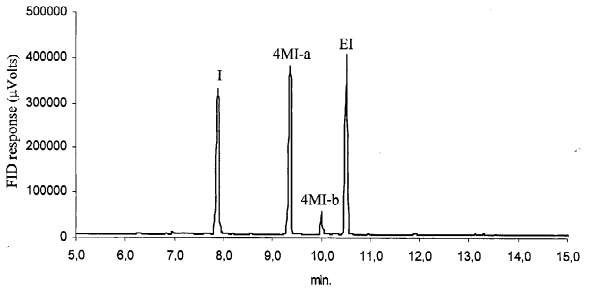

Fig. 1. GC–FID chromatogram of a pure standard solution (250 mg/l) derivatized with ethylchloroformate.

summarises some analytical characteristics obtained with both derivatization conditions.

Due to the complexity of the coffee matrix a purification procedure was necessary in order to achieve the GC analysis of 4MI. The methanolic extract, as used in the preliminary studies described previously, gave complex chromatograms being impossible to find the correct peak identification.

A literature review was done in order to find adequate clean-up procedures. Different methodologies are reported such as solvent extraction [8,9], ion exchange [10–12], or ion-pair extraction [4,13–16]. None of the reported methods using solvent extraction or ion exchange can be considered entirely suitable when the criteria of accuracy, precision, and analysis time are considered. Furthermore, these reported methods do not seem to be specific enough to be used with a complex mixture such as coffee.

Ion-pair extraction with BEHPA was first described by Thomsen and Willumsen [13] and has been the more reported method, with some minor improvements, in the last two decades. Ion-pair extraction seemed very promising for coffee analysis since it proved efficient in other complex matrices,

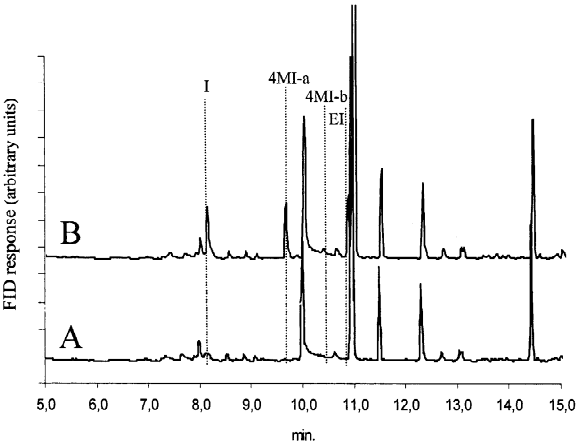

Fig. 2. GC–FID chromatogram of (A) an arabica roasted coffee, and (B) the same sample spiked with 4MI, EI (IS) and I (IS), after derivatization with ethylchloroformate.

such as forage samples and body fluids, where it is described as yielding a clean extract, due to the selectivity in the co-extraction [13].

Ion-pair extraction, performed as described in the Experimental section, was tested on coffee extracts obtained with several solvents, namely methanol, phosphate buffer, 0.1 M H_2SO_4, hot water, and chloroform/ethanol. The highest contents were extracted with methanol, so this solvent was selected.

Fig. 2 represents chromatograms of: (A) roasted coffee, and (B) the same sample spiked with 4MI, EI (IS) and I (IS), after derivatization with ethylchloroformate. Fig. 3 represents the same coffee after derivatization with IBCF.

It can be easily perceived that the peaks corresponding to the compound under analysis, 4MI, are very small and thus difficult to integrate. Also there is some peak interference under the imidazole peak which makes it inappropriate to use as IS.

Table 1
Some analytical characteristics of the derivatives tested

	Retention times (min)		Injection precision (RSD, %)[a]		
	ECF	IBCF	Retention time	Peak area	Peak area/IS
Imidazole	8.2	10.9	0.3	1.1	–
4-(5)Methylimidazole-a	9.7	12.4	0.3	1.4	0.6
4-(5)Methylimidazole-b	10.4	13.1	0.3	1.5	0.7
2-Ethylimidazole	10.9	13.5	0.2	1.2	–

[a] Liquid sampler.

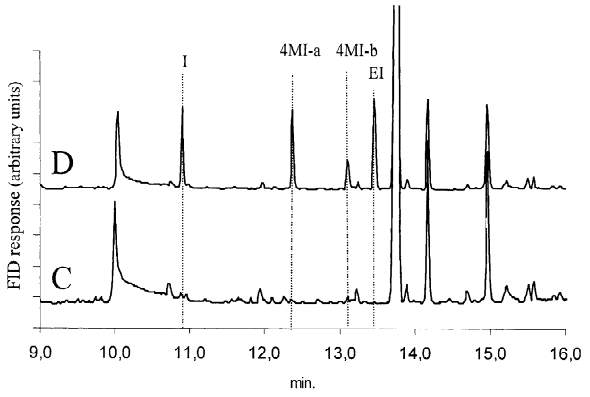

Fig. 3. GC–FID chromatogram of (C) an arabica roasted coffee, and (D) the same sample spiked with 4MI, EI (IS) and I (IS), after derivatization with isobutylchloroformate.

Based on these observations, we have concluded that the determination of 4MI by GC–FID may be possible with other matrices of less complexity but not with those as complex as coffee, even after a selective clean-up procedure such as ion-pair extraction.

3.3. Gas chromatography–mass spectrometry

3.3.1. Quantification method

Full scanning MS, although providing the most unequivocal evidence for identification, does not provide an accurate basis for quantification [17]. Sensitivity can be improved using SIM. We have used multiple-ion monitoring selecting the molecular ions area for each compound quantification, after derivatization with IBCF. Sensitivity could be improved using the main ion m/z 82 for 4MI but the chromatograms were less clear. The other ions were scanned in order to increase selectivity.

Quantification was carried out by the standard addition method. Each sample was analysed without spiking and after a four level spiking, usually from 0.1 to 0.5 mg/kg.

Fig. 4 displays a total ion chromatogram of a torrefacto coffee sample, the corresponding reconstructed molecular ion chromatograms m/z 182 and 196, and the electron impact mass spectra of 4MI-a extracted.

It can be observed that although not completely separated from interfering compounds present, the use of reconstructed chromatograms enables peak separation and accurate area measurement.

The detection limit was estimated at 5.0 μg/kg at a signal-to-noise ratio of 3 for the 4MI-a corresponding to 0.005 ng/μl in the injected extract. The quantification limit was about 0.04 mg/kg.

3.3.2. Reproducibility, recovery and stability

The reproducibility was evaluated by performing five replicate extractions of the same sample. Day-to-day precision was estimated for the same sample over a period of 3 days. The average content was 0.741 mg/kg with a RSD of 5% within extractions and 8% within days. Data obtained from measurements of the recovery efficiency are listed in Table 2, achieving mean results over 98%.

3.3.3. Derivative stability

Once derivatized and stored at 4 °C, the 4MI contained in coffee samples was very stable. The same chloroformic extract analysed after 1 year by different instruments (HP 5890/MSD-5970 and HP 6890/MSD-5973N) gave the same concentration (1.9% RSD).

3.3.4. Chromatographic performance

With both chromatographic columns used, a slight degradation was observed in the peak shape after several injections. The cause for this reduction in the chromatographic performance may be the excess of chloroformate not consumed in the derivatization as described by Fernandes et al. [18]. This problem was not observed in a previous study with chloroformates for amino acid analysis developed by us [5]. Probably the chromatographic column used there (Chirasil) was not affected by the reagent. Attempts to consume this excess of reagent by alkaline methanol or evaporation proved unsuccessful for our compounds as the derivatives were partially lost.

For our purposes, the problem was almost completely resolved by regenerating the column (Bounded Phase Column Rinse Kit, J&W Scientific, USA), although being time consuming.

3.3.5. Sample quantification

The average contents (mg/kg dry wt.) of the coffee samples analysed are reported in Table 3.

To our knowledge, this is the first quantification of

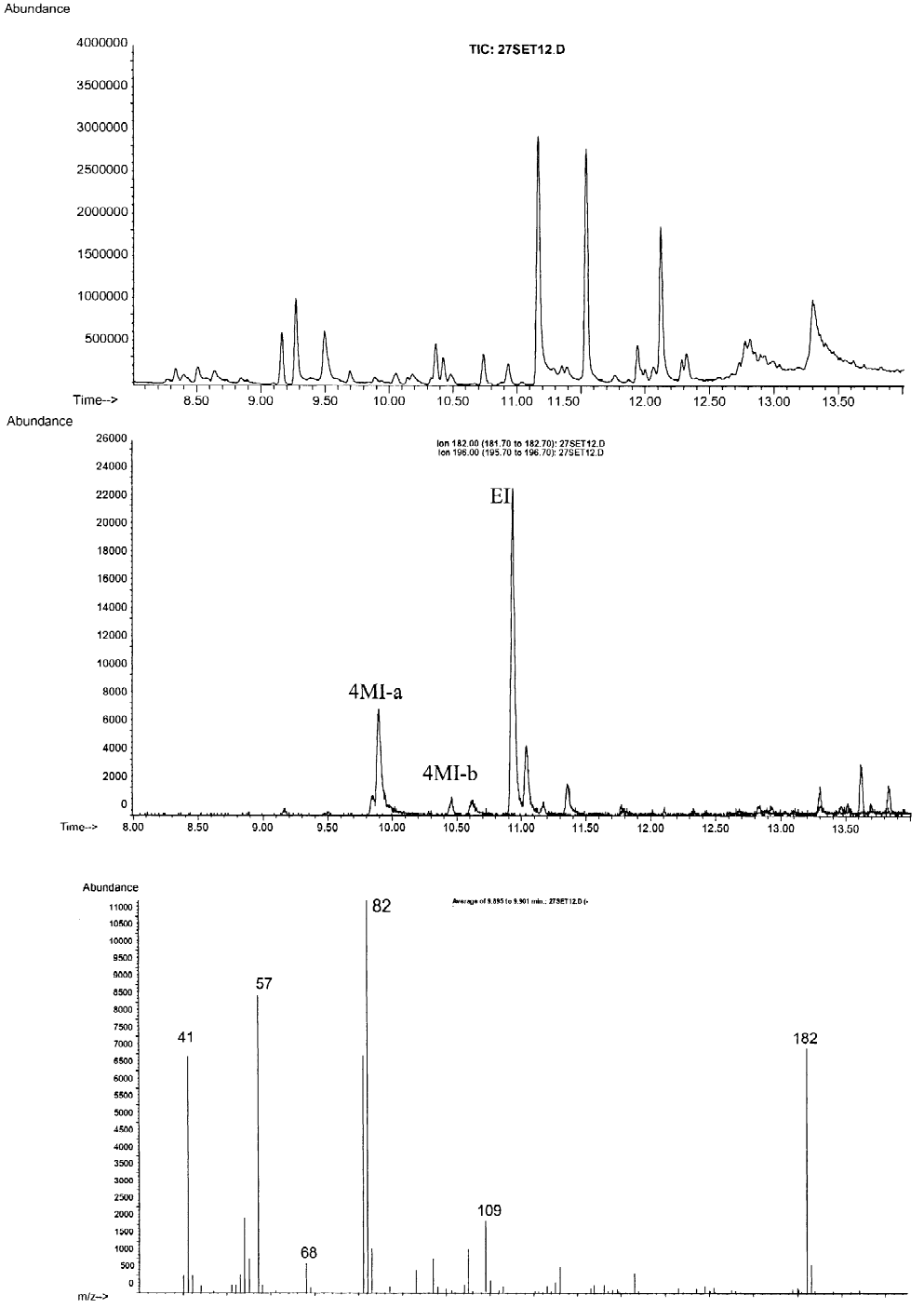

Fig. 4. GC–MS total ion chromatogram of a torrefacto coffee sample (top), the corresponding reconstructed ion chromatograms m/z 182 and 196 (middle), and 4MI-a derivative mass spectra extracted (bottom), after derivatization with isobutylchloroformate.

Table 2
Recoveries from spiked coffee samples

Sample	4MI (mg/kg)			Recovery
	Initial amount	Added	Found	(%)
Roasted arabica	0.702	0.200	0.895	96.4
		0.500	1.201	99.9
Roasted robusta	0.750	0.200	0.956	102.8
		0.500	1.253	100.6
Torrefacto	0.393	0.200	0.594	100.9
		0.500	0.894	100.2
Green arabica	n.d.	0.500	0.432	86.5
		1.000	1.005	100.5

4MI in coffee samples. 4MI was not observed in green coffee and after roasting, the amounts were quite variable ranging from 0.307 to 1.241 mg/kg (dry wt.). The levels found do not seem to be correlated with the coffee variety nor the type of roast, but a larger study is necessary to confirm these possibilities. It would also be interesting to find out if there is any association with the roast type and intensity.

Despite a vast amount of research, evidence to support a direct link of coffee with some diseases has been limited and inconsistent. Overall, the scientific information available supports the safety of moderate consumption [19]. The levels of 4MI present in coffee are extremely low and do not seem to contribute to potential adverse effects.

Table 3
4MI content in several roasted coffees

Sample		mg/kg (dry wt.)
Arabica	Brazil	0.741
Robusta	Ivory Coast	0.801
Torrefacto	100%	0.424
	50%	0.819
	50%[a]	0.307
	50%[b]	0.504
	30%	1.241

[b] Decaffinated.
[a] 100% Arabica.

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