Journal of Food Composition and Analysis 24 (2011) 609–614

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Article

Assessment of 4-(5-)methylimidazole in soft drinks and dark beer

S.C. Cunha [a], A.I. Barrado [b], M.A. Faria [a], J.O. Fernandes [a,*]

[a] REQUIMTE/Department of Bromatology, Faculty of Pharmacy, University of Porto, Rua de Aníbal Cunha 164, 4099-030 Porto, Portugal
[b] CIEMAT/Unidad de Cromatografía, División de Química, Dpto. de Tecnología, Avd. Complutense 22, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 11 January 2010
Received in revised form 4 August 2010
Accepted 13 August 2010
Available online 7 December 2010

Key words:
4-(5-)Methylimidazole
Caramel
Soft drinks
Beer
Ion-pair extraction
GC–MS
Food analysis
Food composition

ABSTRACT

A faster and more robust version of a previously developed method based on ion-pair extraction, acylation with isobutylchloroformate and gas chromatography–mass spectrometry (GC–MS) analysis for determination of 4-(5-)methylimidazole (4-MeI) in soft drinks and dark beer is proposed. The performance of the method was evaluated in terms of linearity (r always > 0.998); recovery (90–101%, 3 levels); and precision (3–8%, 3 levels, $n = 6$). Limits of detection and quantification in the matrices studied were 0.60 μg/L and 2.2 μg/L, respectively. The optimized method was applied to a wide variety of soft drinks (brand name and generic colas, uncarbonated flavor and energy drinks) and dark beers (lager, ales trappist, ales-stout, weissbier). Overall, soft drinks presented higher amounts of 4-MeI (ranging from 37 to 613 μg/L) than those found in the dark beers (ranging from 3 to 424 μg/L), with colas presenting the highest levels. When the different colas analyzed were compared, the 4-MeI levels in generic colas were generally higher than those in brand-name colas. 4-MeI was found in only one of eight energy drinks studied. Based on available consumption patterns, consumer exposure to the maximum 4-MeI given by the soft drinks was 2.3 and 5.7 μg/kg body weight/day, in Europe and the United States, respectively.

1. Introduction

The importance of color perception in the food market can be assessed by popular sayings such as, "We eat first with our eyes". Color additives have been widely used by the food industry to attract consumer attention, stimulate or improve appetite. Among the oldest food color additives are caramel colors, which are brown to brown-black viscous liquids or hygroscopic powders. This group of additives is still used by the food industry today in a wide range of foods and beverages because of its color, flavor and other properties such as stabilization of colloidal systems and prevention of haze formation in beers. Furthermore caramel has emulsifying properties, facilitating the dispersion of water-insoluble materials, retarding flavor changes and preserving the shelf-life of beverages exposed to light (Delgado-Vargas and Paredes-López, 2003).

Caramels are produced by controlled heating of rich carbohydrate sources in the presence of certain reactants such as acids, alkalis, salts, ammonium salts, and sulfites, which results in a complex mixture of compounds. According to the method and reactant used, caramels are classified into four classes: (I) plain caramel E150a; (II) caustic sulfite caramel E150b; (III) ammonia caramel E150c and (IV) sulfite ammonia caramel E150d (Commission Directive 2008/128/EC; JECFA, 2009). The first is used mainly as a flavor additive, while the other three classes are regarded as coloring agents by the food industry.

During the caramelization process a wide range of compounds are generated, some of which are considered "caramel markers." These markers are molecules with low molecular weight such as 4-(5-)methylimidazole (4-MeI) present in class III and IV; 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) present only in class III; and 5-hydroxymethyl-2-furaldehyde (5-HMF), which is present in all four classes of caramel (Pintea, 2008; Delgado-Vargas and Paredes-López, 2003). The occurrence of these markers could be used with authentication purposes; for example, the content of furfural and 5-HMF and their respective ratios have been used to detect whiskey adulteration (Jaganathan and Dugar, 1999).

The presence of these minor caramel components in most foods and beverages, however, can be hazardous to humans because of toxicity. 4-MeI is a neurotoxic agent (Patey et al., 1985) and some *in vitro* studies have shown its capability to inhibit the cytochrome P450 isoenzyme which catalyses the oxidation of many known or suspected carcinogens of low molecular mass in the human liver (Hargreaves et al., 1994). Furthermore, a recent toxicological study conducted by the National Institute of Environmental Health Sciences of USA (Chan et al., 2008) showed that 4-MI can induce alveolar/bronchiolar adenoma and carcinoma in male and female mice. THI in turn has been related to immunosuppressive effects (Reeve et al., 1993), while 5-HMF is considered an irritant to the eyes, upper respiratory tract, skin and mucous membranes.

* Corresponding author. Tel.: +351 222078910; fax: +351 222003977.
E-mail address: josefer@ff.up.pt (J.O. Fernandes).

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doi:10.1016/j.jfca.2010.08.009

The Codex Alimentarius of the World Health Organization (WHO) and the European Union (EU) have established a maximum of 250 mg/kg for 4-MeI, for caramels class III and IV, and a limit of 10 mg/kg for THI for caramels class III (WHO, 1971; Document III/5218/94-EN-Rev, 1995). Until now, no limit levels have been established for the presence of 4-MI in foodstuffs. Monitorization is nevertheless necessary in order to ensure that the caramel added to the foods and beverages are declared in the label, to estimate the levels of caramel added, and to guarantee that human dietary intakes are within acceptable levels.

Several methods have been developed to determine 4-MeI based on thin layer chromatography (TLC) (Rabe et al., 1988), fluorimetry (Gutierrez et al., 1986), capillary electrophoresis (Ong et al., 1994) or high performance liquid chromatography (HPLC) coupled with ultraviolet light (UV) (Thomsen and Willumsen, 1981; Coffey et al., 1997). However, these methods require a labor- and time-consuming sample pre-treatment and have poor sensitivity. In recent years more sensitive methods have been published, based mainly on mass spectrometry (MS) as a detection technique, coupled with a chromatographic step either by liquid chromatography (LC) (Klejdus et al., 2003, 2006; Lojková et al., 2006) or gas chromatography (GC), the latter after derivatization of the analytes (Fernandes and Ferreira, 1997; Casal et al., 2002). The main advantage of the recent LC–MS methods proposed by the group of Kubán is the possibility to analyze simultaneously 4-MeI and THI without derivatization (Klejdus et al., 2003, 2006; Lojková et al., 2006). Notwithstanding the high selectivity achieved by this technique, the methods include a previous tedious solid-phase extraction (Klejdus et al., 2006) or supercritical fluid extraction (Lojková et al., 2006).

The use of GC–MS methods based on ion pair-extraction with bis-2-ethylhexilphosphate (BEHPA) and isobutylchloroformate derivatization has been successfully applied for determination of 4-MeI at trace levels in caramel and coffee (Fernandes and Ferreira, 1997; Casal et al., 2002). The application of this method to other matrices seems of great interest because of the selectivity and sensibility obtained. However, some problems related to degradation of the columns following injection of chloroformic extracts containing excess of isobutylchloroformate (Casal et al., 2002; Fernandes et al., 2001) have prevented widespread application to other matrices.

The main objectives of this work were: (i) to improve some features of the previously developed method, based on ion-pair extraction with isobutylchloroformate derivatization and GC–MS analysis, to increase its ruggedness and reliability when applied to other food matrices; (ii) to conduct a survey on the presence of 4-MeI in soft drinks and dark beer samples; and (iii) to assess the 4-MeI intake from European and American consumers, based on 4-MeI levels obtained from soft drinks and the available consumption data.

2. Materials and methods

2.1. Reagents and solutions

4-(5-)Methylimidazole (purity ≥ 99%) and 2-ethylimidazole (2-EI, purity ≥ 98%) were purchased from Sigma (West Chester, PA; USA) and from Aldrich (Steinheim, Germany), respectively. *Bis*-2-ethylhexylphosphate (BEHPA; purity ≥ 98%) was from Aldrich and isobutylchloroformate (IBCF; purity ≥ 99%) was purchased from Sigma. Isooctane and acetonitrile (MeCN) both of LiChrosolv quality were purchased from Merck (Darmstadt, Germany). Pyridine (over molecular sieve, purity > 99.8%), acetic acid (purity > 99.7%) and isobutanol (purity > 99.8%) were purchased from Fluka (Neu-Ulm, Germany). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate, used to prepare phosphate buffer, were purchased from Sigma. All the other reagents were analytical grade.

Ultrahigh purity He (helium) for GC–MS and N_2 (nitrogen) for solvent evaporation were obtained from Gasin (Maia, Portugal).

2.2. Standards

A stock solution of 4-MeI (2 g/L) was prepared by dissolving the compound in 0.1 M HCl. An intermediate standard solution (2 mg/L) was prepared from the stock solution by appropriate dilution in 0.1 M HCl. A working 1 g/L solution of the 2-EI used as internal standard (I.S.) was also prepared in 0.1 M HCl. All the solutions were kept at 4 °C when not in use. Linearity was studied using matrix-matched calibration by analyzing blank samples (free of 4-MeI) spiked at six concentration levels, in order to obtain concentrations ranging from 20 to 750 μg/L. The concentration of the samples was obtained by the internal standard method.

2.3. Sampling

A total of 30 samples of soft drinks comprising 16 colas, 8 energy drinks, 6 uncarbonated flavor drinks and 1 carbonated guarana were randomly purchased in local supermarkets. A total of 2 colas were acquired in supermarkets in Spain and 3 colas and 1 energy drink were obtained in supermarkets in France. A total of 20 samples of dark beers were also purchased in local supermarkets. All the samples were stored at room temperature (±20 °C) protected from light and opened only on the moment of analysis.

2.4. Sample preparation

2.4.1. Ion-pair extraction and derivatization of 4-MeI

4-MeI was extracted from the samples using a procedure based on a previously described methodology (Fernandes and Ferreira, 1997) with some modifications; the analysis scheme is shown in Fig. 1. An aliquot of 25 mL of homogenated sample added with 50 μL 2-EI (I.S.) at 1 g/L was introduced in a 50 mL glass centrifuge tube and concentrated in a Büchi Rotavapor model RE 111 with a 461 water bath (Flawil, Switzerland) at 60 °C, to about 5 mL (Fig. 1). Then, 1 mL of the sample concentrate was placed into a mL vial, and the pH of the mixture adjusted to 6.6 by drop-wise addition of concentrated potassium hydroxide solution followed by addition of 1 mL of phosphate buffer. The mixture was extracted with 2 mL of 0.1 M BEHPA in chloroform, through hand mixing for 1 min and vortexing for 2 min. After a centrifugation step at 5000 rpm for 5 min, a 1.8 mL portion of the chloroform phase (bottom layer) was further removed to a second vial which contained 1.0 mL of 0.1 M HCl. The mixture was again mixed by hand for 1 min and centrifuged at 5000 rpm for 1 min. Then, a 250 μL aliquot of the aqueous phase (upon layer) was transferred to a reaction vial and derivatized with a 250 μL of MeCN–isobutanol–pyridine (50:30:20, v/v) and 30 μL (15 + 15 μL) of IBCF through a brief shaking (15–30 s). Finally, 500 μL of a 1.0 M aqueous sodium bicarbonate solution and 500 μL of isooctane were added into the mixture and after a brief shake of 4–5 s, the bottom layer was transferred to an autosampler vial and 2 μL were injected into the GC–MS system.

2.4.2. Apparatus and GC–MS conditions

The determination of 4-MeI was performed on an Agilent (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port, an inert 5975B mass selective detector with electron impact (EI) ionization chamber, and a 7683B Series injector/autosampler.

The GC separation was conducted with a DB-5ms column (15 m × 0.25 mm I.D. × 0.25 μm film thickness; J&W Scientific,

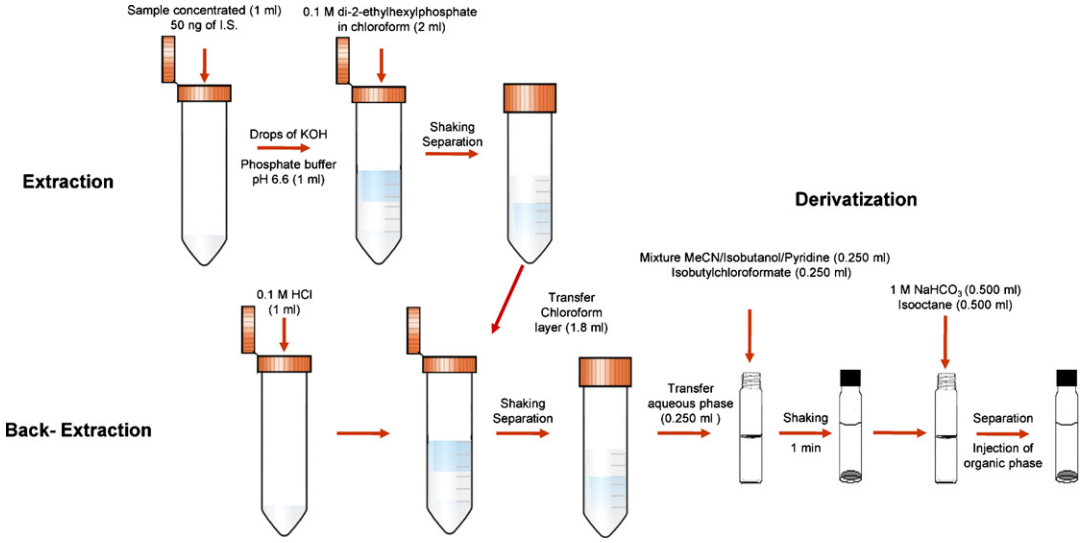

Fig. 1. Sample preparation scheme used in this study.

Folsom, CA, USA). Helium was the carrier gas with a constant pressure of 80 kPa. The injection was made in splitless mode at 270 °C. The oven temperature program was as follows: 80 °C held for 1.0 min, ramped to 280 °C at 30 °C/min and held for 1.83 min. The MS transfer line temperature was held at 280 °C. Total run time was 9.5 min.

Mass spectrometric parameters were set as follows: electron impact ionization with 70 eV energy; ion source temperature, 230 °C; MS quadrupole temperature, 150 °C and solvent delay, 2 min. The MS system was routinely set in selective ion monitoring (SIM) mode and 4-MeI was quantified based on peak area using one target and three qualifier ion(s). Complete SIM parameters and retention times of the analytes are shown in Table 1. Agilent Chemstation was used for data collection/processing and GC–MS control.

2.5. Statistical analysis

The analysis was carried out with SPSS for Windows 17.0 (SPSS Corporation, Chicago, IL). Kolmogorov–Smirnov test was used to verify parametric or nonparametric characteristic of data. To evaluate the difference between samples a nonparametric test (Mann–Whitney *U*-test) was chosen due to sample size and non-normal distribution. Statistical significance was assumed if a null hypothesis could be rejected at $p < 0.05$.

3. Results and discussion

3.1. Optimization of sample preparation and chromatographic conditions for analysis of 4-MeI

Ion-pair extraction provides the extraction of ionizable compounds into an organic phase as an ion-pair by addition of a suitable ion with opposite charge (Carson, 2000). An ion-pair extraction procedure has been successfully applied in extraction of 4-MeI from ammonia caramel colors (Thomsen and Willumsen, 1981; Fernandes and Ferreira, 1997), and coffees (Casal et al., 2002), using BEHPA as ion-pair reagent and chloroform as organic solvent. The procedure proved to be quite selective and effective in the extraction of 4-MeI and similar imidazolic compounds from complex matrices. Given the low amounts of 4-MeI expected in the samples under study, a previous concentration step was performed, in order to improve detection and quantification limits.

The 4-MeI extracted by ion-pair was then acylated with IBCF in presence of the acetonitrile–isobutanol–pyridine mixture, in order to improve the thermal stability of the analyte and its chromatographic behavior. The derivatives were subsequently extracted with isooctane instead of chloroform used in the previous works. The use of isooctane totally solved the problems of rapid deterioration of chromatographic performance (pronounced tailing and gradual decrease in peak areas) that was previously verified after several injections of IBCF derivatives in chloroform in similar DB-5 MS columns (Fernandes et al., 2001; Casal et al., 2002). After more than 300 injections made with standard and sample extracts in the same column no visible degradation of the peaks shape and peaks area was observed. The use of a short capillary column (15 m) allowed the reduction of retention times corresponding to the peaks of interest into about 4 min when compared with our previous works, without lost of resolution or sensibility.

Fig. 2 shows a chromatogram of 4-MeI standard at 250 μg/L and a positive sample with 399 μg/L of 4-MeI obtained using the described procedure. As observed two peaks corresponding to 4-MeI (a and b) were detected. This observation was valid either for standards or samples and it was already described by Fernandes and Ferreira (1997). It is probably due to the natural tautomerism characteristic of substituted imidazoles (Worth et al., 1989; Hasegawa et al., 2000). This was, however, no constraint for a correct quantification of the compound, since the sum of peak areas was used.

Table 1
MS conditions for 4-[5-]methylimidazole [(4-)MeI] and internal standard (I.S.) analysis (start times of windows and ions selected in SIM mode, quantification ions in bold).

Compounds	t_R (min)	Time windows (min)	Data acquisition rate (scans/s)	SIM ions (*m*/*z*)
4-MeI (a)	3.42			**182**, 82, 109, 81
4-MeI (b)	3.72	2.0–9.5	3.75	**182**, 82, 68, 81
2-EI (I.S.)	3.76			**196**, 95, 123, 81

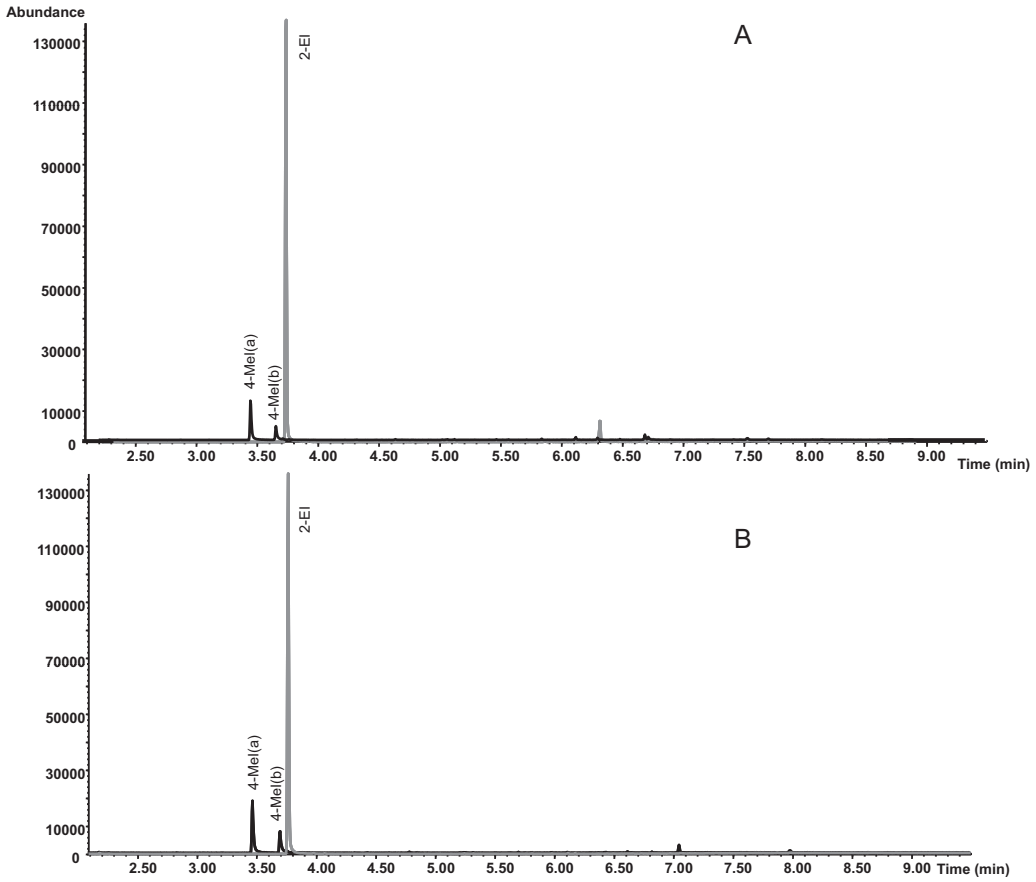

Fig. 2. Chromatograms in SIM mode of a spiked blank soft drink [250 μg/L of 4-MeI (total a and b) and 1 mg/L of I.S. (2-EI)] and a positive generic cola sample [399 μg/L of 4-MeI (total a and b) and 1 mg/L of I.S. (2-EI)] obtained by the optimized GC/MS method. Ion 182 for 4-MeI (a and b) and ion 196 for 2-EI.

3.2. Method performance

3.2.1. Linearity

The possible matrix effect on the chromatographic response was preliminary assayed. Therefore the slopes of the calibration curves obtained from aqueous standard solutions were compared with those obtained in matrix-matched standards (standards added to blank samples). The slopes values were 0.3185 for the aqueous solutions and 1.0170 and 1.0478 for soft drinks and beer, respectively. This enhancement response for 4-MeI was observed previously for other analytes in distinct kind of matrices (Cunha et al., 2009; Poole, 2008). It can be related with the properties of the analyte itself as well as the presence of other ionizable compounds present in the extract. Although the slope between both matrices are not statistically different ($p > 0.05$) indicating that the selected matrices do not have any significant influence on the extraction procedure, matrix-matched calibration solutions of either blank soft-drinks and blank beers were used for quantification purposes. Thus the linearity of the method was several times tested using the referred matrix-matched calibration solutions with six concentrations (20, 50, 100, 250, 500 and 750 μg/L), prepared as described in Section 2. Calibration curves were constructed by plotting the analyte/I.S. peak areas ratio obtained against the concentration values. The results obtained had a quite good linearity with correlation coefficients always higher than 0.998 in the two distinct matrices studied.

3.2.2. Recovery and precision

Recovery and precision were determined on blank samples of soft drinks spiked with 4-MeI at three concentration levels, being each test performed six times, as described in Section 2. As shown in Table 2 the average of recoveries ranging from 90 to 101%. The precision measured by the relative standard deviation (RSD) ranging from 3 to 8%. The results reported provide evidence that the optimized method guarantees that 4-MeI can be properly quantified.

3.2.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of the method was determined by successive analyses of chromatographic sample extracts with decreasing amounts of the compounds until a 3:1 signal-to-noise ratio was reached, whereas the LOQ was determined considering a signal-to-noise ratio 10:1. In this study because the quantification was made with the sum of the peak areas of 4-MeI (a) and 4-MeI (b), the size of the latter was the limiting factor. The LOD and LOQ were 0.60 μg/L and 2.2 μg/L, respectively. These values are lower than those reported in the previous paper for caramel (Fernandes and Ferreira, 1997).

3.3. Analysis of 4-MeI in soft drinks and dark beers

The content of 4-MeI in various soft drinks (white and brand-name colas, energy drinks and uncarbonated flavor) was determined

Table 2
Average recoveries (%) and precision (%RSD, relative standard deviation) obtained in spiked soft drink samples using the developed method.

Level added (μg/L)	Recovery (%)	Precision (%RSD)
50	101	8
250	90	5
750	92	3

Table 3
Level of 4-[5-]methylimidazole [(4-)MeI] (average and % relative standard deviation $n = 2$) in soft drinks and difference between the types of samples analyzed.

Type	Geographic origin	Label information	4-MeI μg/L (%RSD)
Brand cola[a,b]	Portugal	E150d	248 (1)
		E150d	228 (2)
		E150d	249 (1)
		E150d	340 (1)
		E150d	243 (2)
		E150d	287 (1)
		E150d	214 (2)
		E150d	372 (1)
		E150d	360 (1)
	Spain	E150d	416 (1)
		E150d	270 (1)
	France	E150d	188 (2)
		E150d	333 (1)
White label cola[b]	France	E150d	219 (2)
	Portugal	E150d	454 (1)
		E150d	305 (1)
		E150d	255 (1)
		E150d	430 (1)
		E150d	399 (1)
		Caramel	406 (1)
		E150d	613 (1)
Energy drink[a]	Portugal	Caramel	n.d.
		Caramel	n.d.
		E150b	n.d.
		E150d	n.d.
		E150d	n.d.
		E150d	n.d.
		E150d	37 (12)
	France	Caramel	n.d.
Carbonated guarana[b]	Portugal	E150d	438 (2)
No carbonated flavor[a,b]	Portugal	E150d	43 (10)
		E150d	42 (8)
		E150d	71 (6)
		–	n.d.
		Caramel	n.d.
		E150d	n.d.

–: not mentioned; n.d.: not detected. [a,b]Means in groups without common letters are significantly different ($p < 0.05$). E150d (sulfite ammonia caramel).

in duplicate following the methodology described. The results obtained and the relevant information reported in the label of each sample analyzed are shown in Table 3. 4-MeI was found in 26 of 36 soft drinks analyzed, being the levels found extremely variable, ranging from 37 to 613 μg/L. The higher values were found in generic colas, with levels ranging from 219 to 613 μg/L, a mean of 385 μg/L ($n = 8$). Brand-name colas presented slightly lower levels, ranging from 188 to 416 μg/L, with a mean of 288 μg/L ($n = 13$). The 4-MeI contents in brand-name colas were slightly lower than those reported by our group ten years ago for similar samples, with levels ranging from 380 to 715 μg/L, in 10 similar samples (Fernandes et al., 1997). The difference found between both studies can be justified by the number of samples analyzed and probably by the improvements that naturally have taken place at the level of process and control systems to guarantee the quality and safety of the final product. Regarding the geographical origin of the colas no significant differences were found between the samples of the three countries analyzed, suggesting that colas technological process is not considerably different.

On the contrary to what was verified in colas samples where all samples were positive, in the energy drinks only 1 of 8 samples analyzed shown 4-MeI, in a concentration of 37 μg/L. The carbonated guarana present a value of 4-MeI (438 μg/L) near of the colas, even though presenting lower color intensity from a visual point of view. To the uncarbonated flavor drinks, 4-MeI was found in 3 of 6 samples analyzed, with levels ranging from 42 to 71 μg/L. These results are in accordance with those previously reported for carbonated beverage by Fernandes et al. (1997).

Table 4
Level of 4-[5-]methylimidazole [(4-)MeI] (average and % relative standard deviation $n = 2$) in dark beers and difference between the types of samples analyzed.

Type	Geographic origin	Label information	4-MeI μg/L (%RSD)
Lager-Dark[a]	Portugal	Caramel	325 (1)
		–	170 (2)
		–	172 (2)
		–	77 (3)
		–	n.d.
		–	n.d.
	Spain	–	8 (10)
	Germany	–	3 (9)
		–	39 (4)
		–	n.d.
Ales-trappist[a]	Belgium	–	20 (7)
		–	150 (2)
		–	424 (1)
Ales-stout[a]	Irland	–	21 (7)
		–	18 (8)
	Portugal	E150c	140 (2)
		Caramel	142 (2)
Weissbier-dark[a]	Germany	–	n.d.
		–	n.d.
		–	3(8)

–: not mentioned; n.d.: not detected. E150C (ammonia caramel).

Results found for 4-MeI in dark beers samples are summarized in Table 4, as well as all important information reported in the labels. The 4-MeI was found in 15 of the 20 samples analyzed, with great variation. Some were either free from or had a negligible amount of 4-MeI the levels ranging from 3 to 424 μg/L. However, the addition of caramel color during the brewing process is not generally mentioned in the label (see Table 4). The concentrations found in this study were higher than those reported by Klejdus et al. (2006), with levels ranged from 1.6 to 28 μg/L. This discrepancy may be partly attributed to the origin and number of the samples analyzed. Concerning the influence of the beer type in the 4-MeI content, no significant ($p > 0.05$) differences were found between the samples belonging to the four beer categories.

3.4. Exposure to 4-MeI via ingestion

It is known that the human exposure to 4-MeI may have adverse health outcomes, e.g. neurotoxic effects (Patey et al., 1985; Delgado-Vargas and Paredes-López, 2003). This fact assumes particular importance taking in consideration the steady rise of soft drink consumption in the recent last years worldwide. The major consumption of soft drinks in the world occurs in the USA, with 216 L/year per capita in 2002, comparing with 204 L/year per capita in 1999 (Hawkes, 2002), with colas representing more than 70%. In what concern Western Europe the consumption of soft drinks was only 83.2 L/year per capita in 1999 (Hawkes, 2002). European statistics from a different source referring to 2002 (Hawkes, 2002) show identical whole values, although with large variations between different countries, from 126 L/year per capita in Ireland to 37.2 L/year per capita in France (http://www.nationmaster.com).

Preliminary assessment of consumer's exposure to 4-MeI in soft drinks was calculated by the estimated daily intake (EDI). The EDI expressed in μg/kg body weight/day was obtained as the result of the soft drink consuming plus the maximum amount of 4-MeI found in soft drinks (613 μg/L). The consumption values considered were the mentioned above for 1999, and a human body weight of 60 kg was assumed. The estimated daily intake of 4-MeI in Europe was 2.3 μg/kg body weight/day and 5.7 μg/kg body

weight/day in USA for soft drinks. The total human daily intake of 4-MeI is obviously higher because other sources of the compound should be considered (baked goods, confectionery, extruded breakfast cereals, instantaneous soups, dark beers, etc.).

It is difficult to compare the results obtained here with those of other monitoring programs from other countries, because they are scarce and mostly outdated. Furthermore, the only acceptable daily intake (ADI) established for 4-MEI by WHO is for caramel colors produced by ammonia process (0–100 mg/kg body weight/day).

4. Conclusions

A modified method was evaluated for determination of 4-MeI in soft drinks and dark beers. The proposed method based on ion-pair extraction, acylation with isobutylchloroformate and GC–MS analysis maintained all of the accurately characteristics of the original method with additional improvements in robustness and sensibility. The method was used for a survey on the presence of 4-MeI in several types of soft-drinks (colas, carbonated and uncarbonated flavor drinks and energy drinks) and dark beers. The study reveals that 4-MeI is ubiquitous in colas with levels on the order of some hundreds of milligrams per liter. Generic colas presented slightly higher concentrations than brand-name products. Most of the dark beers analyzed and a carbonated guarana soft drink also showed substantial levels of the compound. Instead, only one of 8 energy drinks analyzed was positive for 4-MeI. Based only on cola consumption, the mean per capita daily intake of 4-MeI was estimated to range from 2.3 to 5.7 μg/kg body weight/day, in Europe and USA, respectively. These values vary widely according to individual consumer habits, and in some cases they probably reach levels of concern for human health.

Acknowledgments

This research was supported by grant from the FCT project "PTDC/AGR-ALI/101583/2008" and COMPETE FSE/FEDER. S.C.C. is grateful to "POPH-QREN-Tipologia 4.2, Fundo Social Europeu e Fundo Nacional MCTES". M.A. Faria is grateful to FCT for the Grant SFRH/BPD/20725/2004.

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