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Original Article

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

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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.

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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,