Quantification of 4-Methylimidazole in soft drinks, sauces and vinegars of Greek market using two liquid chromatography techniques

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Abstract

The substance 4-methylimidazole (4-MEI) has raised several concerns regarding its toxicity to humans, although no harmonized classification has yet been decided. The regulatory limits for food products set by various authorities in Europe and the USA differ considerably. The purpose of the present study is to compare two liquid chromatography techniques in order to determine the levels of 4-MEI in food products from the Greek market and roughly estimate the possible exposure and relevant health risk for the consumers.

A total of thirty-four samples (soft drinks, beers, balsamic vinegars, energy drinks and sauces) were collected and analyzed. The quality parameters for both analytical methodologies (linearity, accuracy, inter day precision, recovery) are presented. No detectable levels of 4-MEI are found in beers and soft drink samples, other than cola type. On the other hand, 4-MEI was detected in all cola type soft drinks (15.8–477.0 ng/ml), energy drinks (57.1%, 6.6–22.5 ng/ml) and vinegar samples (66.7%, 9.7–3034.7 ng/ml), while only one of the sauce samples was found to have a detectable level of 17.5 ng/ml 4-MEI.

1. Introduction

The substance 4-Methylimidazole (4-MEI) is a heterocyclic compound, which supposedly does not occur as a natural product since it is produced via heating carbohydrates with ammonium compounds (Chappel and Howell, 1992). It is used as an agent in the manufacture of pharmaceuticals, photography as well as in the food industry. It has been detected in cigarette smoke (Zhu et al., 2015), roasted foods and coffee (Casal et al., 2002), grilled meats and coffee (IARC Monographs, 2012), caramel color additives (Thomsen and Willumsen, 1981) and in a wide range of foods, sauces, vinegars, beers and soft drinks (Cunha et al., 2011; Lim and Shin, 2013; Fernandes and Ferreira, 1997).

According to the method used for the preparation of caramel colors, they are classified in four groups: E150a, E150b, E150c and E150d. 4-MEI is considered as market for the caramel colors E150c (Ammonia Caramel) and E150d (Sulphite Ammonia Caramel) (Cunha et al., 2011). Ammonia caramel (E150c) is mostly used in bakery products, sauces, soups, beers and vinegars while ammonium-sulfite caramel (E150d) is commonly found in soft drinks, pet food and soups (IARC Monographs, 2012). In caramel colors

Abbreviations: IS, internal standard; LC-MS, liquid chromatography-mass spectrometry; 4-MEI, 4-methylimidazole; CDL, curved desolvation line; ESI, electrospray ionization; SIM, selected ion monitoring; LOD, limit of determination; LOQ, limit of quantification; SD, standard deviation; TOX.JS, Automated and fast prominence HPLC system with photodiode array detector; HPLC, high performance liquid chromatography; NOAEL, no-observed-adverse-effect level; NSRL, No Significant Risk Level.

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manufactured from 2008 to 2010 according to recent analytical data from the industry, the levels of 4-MEI regarding E150c (103 samples) has a mean value of 41.6 mg/kg, with a range of 5–140 mg/kg and E150d, for single strength, has a mean value of 102.4 mg/kg with a range of 48–183 mg/kg and for double strength, a mean value of 88.3 mg/kg with a range of 23–147 mg/kg (EFSA, 2011).

According to the Regulation (EC) No 1333/2008 the use of caramel color has been restricted to E150 a-d only in vegetables and red fruits, excluding olives, vinegar, oil and brine. The same Regulation limits the use of E150 a-d only in beers and malt/Tafelbier/Table beer (original wort content less than 6%), brown ale, porter and stout and old ale. The use of E150a-d is banned in fruit spirits obtained by maceration and distillation of Geist, London Gin, Sambuca, Maraschino, Marrasquino or Maraskino and Mistrà. Whisky, whiskey can only contain E 150a.

4-MEI does not have a harmonized classification under Regulation (EC) No 1272/2008. In the inventory of the European Chemicals Agency (ECHA), almost 240 notifiers have classified the substance, but not as a mutagen, whereas more than 60% of the notifiers classify 4-MEI as a carcinogen category 2 (suspected of causing cancer). In addition, 4-MEI is reported as a neurotoxic agent (Cunha et al., 2011; Patey et al., 1985), “probably carcinogenic” in mice and female rats at high doses (National Toxicology Program, 2007; Lim and Shin, 2013) and the International Agency for Research on Cancer (IARC) classifies it as a “possibly carcinogenic to humans” (group 2B) (Schlee et al., 2013).

The World Health Organization (WHO) and the European Union have established a maximum level of 4-MEI, for the caramel class E150c and E150d, to be set at 250 mg/kg (Cunha et al., 2011).

According to the NTP (National Toxicology Program, 2007) report, an increased incidence of lung tumors was recorded in mice exposed to high levels of 4-MEI (>10.000 times higher than the human dietary intake) (Morita and Uneyama, 2016). Based on a negative evaluation of genotoxicity of 4-MEI, the European Food & Safety Authority (EFSA) has established a NOAEL of 80 mg/kg bw/day. On the other hand, CEPA (California Environmental Protection Agency) has adopted concerns regarding the mutagenic properties of 4-MEI and consequently has adopted a “No significant Risk Level” (NSRL) for 4-MEI to be 29 μg/day (propositions 65), which is defined as the daily intake level posing a 10^-6 lifetime risk of cancer (Morita and Uneyama, 2016; CEPA, 2011) (Table 1).

Gas and liquid chromatography coupled with mass spectrometry methodologies have been developed and various techniques of separation and extraction have been applied for 4-MEI determination, as ion-pair extraction (Thomsen and Willumsen, 1981), headspace solid phase microextraction (Lim and Shin, 2013) or liquid solvent extraction (Casal et al., 2002).

The aim of the present study is to compare two analytical liquid chromatography techniques for the direct detection of 4-MEI in food products from the Greek market and roughly estimate the exposure of the Greek consumers to 4-MEI.

2. Materials and methods

2.1. Reagents

Methanol (LC-MS grade), 4-MEI (98%) and ammonium formate (>99%) were purchased from Sigma (Sigma Aldrich Co. 3050 Spruce street, St Louis, USA) while ultrapure water was produced by a Direct-Q 3UV water purification system (Merck, Germany).

2.2. 4-MEI chromatographic analysis by TOX.IS

A Shimadzu Prominence TOX.IS HPLC system (Shimadzu, Duisburg, Germany) was used for the detection of 4-MEI in food products, equipped with a pump system, a solvent degasser, an autosampler with a 2 ml loop, an oven and a diode-array detector. The extraction column (SPE-column), guard column and the two analytical columns with guard cartridges (ClinPrep) were obtained from RECIPe-Chemicals (Munich, Germany). The oven was set to 40 °C, the detection was performed from 190 to 370 nm and the mobile phase flow rate was 2.0 ml/min. The separation was achieved using solvents provided from RECIPe-Chemicals: ClinRep, mobile phase solvent A, ClinRep, mobile phase solvent B, ClinRep, mobile phase solvent AB and ClinRep, SPE-Washing solution for TOX.IS. The total run time was 44 min. Under the above conditions the retention time of 4-MEI was 21.8 min. Typical chromatograms of standard and spiked solutions of 4-MEI and chromatograms of both negative and positive soft drink samples (C = 190 ng/ml) received by TOX.IS is shown in Fig. 1.

2.3. 4-MEI chromatographic analysis by LC-ESI-MS

A liquid chromatography-mass spectrometry system (Shimadzu) was used for the quantification of 4-MEI. As mobile phase, a gradient of 5 mM ammonium formate in water (solvent A) and methanol (solvent B) were selected and the flow rate was 0.6 ml/min: starting at 95% solvent B (0.1 min) to 1% solvent B (10.0 min) and finally 95% solvent B (10.1 min) for 7 min 4-MEI was eluted at 6.2 min using a 250 × 4.6 mm, 5 μm, Discovery C18 column at 20 °C. For the detection and quantification of 4-MEI a mass spectrometer

Table 1

Regulatory bodies | Decision | Reference
--- | --- | ---
CEPA, 2011 | Since the lung is a primary target tissue in mice, it should be used as a source tissue for in vitro metabolic activation system. NSRL of 29 μg/day based on the non-threshold mechanism. | California Environmental Protection Agency (CEPA), 2011.
EFSA, 2011 | No DNA mutations, lung tumors in mice are spontaneous. NOAEL 80 mg/kg bw/day. Application of the threshold mechanism. | European Food Safety Authority (EFSA), 2011.

* NSRL, No Significant Risk Level.
coupled with an ESI interface was used, in the positive SIM mode, with ions m/z 82.95 and 84.05. The CDL and the heat block temperatures were 200 °C. A typical LC-MS chromatogram of standard solution (50 ng/ml), spiked solution (500 ng/ml), positive cola type sample for 4-MEI (472.7 ng/ml) and energy drink sample (negative for 4-MEI) is shown in Fig. 2.

2.4. Sampling

Thirty-four (34) samples (15 soft drinks, 14 cola type and 1 no cola type, 7 energy drinks, 2 beers, 6 vinegars and 4 sauces) were collected from the Greek market during 2014 and analyzed. Throughout the study, all samples were stored in a dry dark place, at room temperature.

2.5. Stock, working solutions and spiked samples

Stock solution of 4-MEI, at a concentration of 1000 ppm was prepared in methanol and stored at –20 °C. Dilutions of the stock solution was applied and the following standard working solutions were prepared 0, 100, 250, 500 and 2500 ng/ml. Neostigmine (25 μg/ml) was used as internal standard. The working solutions of 4-MEI for LC-MS had a concentration level of 0, 25, 50, 100, 250, 500 and 1000 ng/ml.

Five spiked solutions were prepared using blank no cola type drinks to final levels 0, 100, 250, 500 and 1000 ng/ml of 4-MEI for TOX.IS analysis and concentration levels of 0, 25, 50, 100, 250 and 500 ng/mg for LC-MS analysis.

2.6. Sample preparation

The samples, for TOX.IS analysis, were prepared by mixing 0.25 ml of each sample, 0.25 ml of water and 0.5 ml of IS. Spiked solutions and samples were vortexed, homogenized and centrifuged for 5 min at 14,000 rpm. The injection volume of each sample was 950 μl. For LC-MS analysis, each sample was diluted by a fivefold factor (1:5) using 5 mM ammonium formate in water (Schlee et al., 2013), following a vortex for 1 min and centrifugation for 4 min at 14,000 rpm. A portion of 10 μl of the above was injected for analysis in the LC-MS system.

3. Statistical methods-risk definitions

The measures of central tendencies used to describe continuous variables were mean, median and minimum-maximum values, while standard deviation was used as a measure of dispersion. Comparison of continuous variables between groups was assessed using non-parametric tests such as Mann-Whitney (for two groups) and Kruskal-Wallis (for more than 2 groups). IBM Statistics 21.0 was used for the statistical analysis and a level of 0.05 was set for accepting (rejecting) the null hypotheses.

4. Results

4.1. Method validation

4.1.1. Linearity and limit of quantification

Standard curves were developed after the analysis of the standard solutions of 4-MEI. The instrument response was linear in the range from 0 to 1000 ng/ml ($r^2 = 0.999$ for TOX.IS and $r^2 = 0.994$ for LC-MS). Spiked curves were constructed and used for the quantification of 4-MEI in the actual samples. The concentration ranges were from 0 to 1000 ng/ml for TOX.IS and from 0 to 500 ng/ml for LC-MS with linear curves ($r^2 = 0.996$ for TOX.IS and $r^2 = 0.993$ for LC-MS, respectively).

The limit of determination (LOD) and quantification (LOQ) of LC-MS were determined using the lowest concentration (25 ng/ml) spiked sample and defined as the peak that gave a signal to noise ratio > 3 and 10, respectively. LOD and LOQ values were determined to be 4.0 ng/ml and 1.2 ng/ml for the LC-MS. The LOQ value for TOX.IS was determined by injected decreased concentration solutions and determined to be 25 ng/ml (Table 2).
4.1.2. Recovery and ion suppression

The recovery of the procedure was evaluated by analyzing spiked samples at different concentrations (from 50 to 500 ng/ml). The mean % recovery of 4-MEI was found to be 84.2 ± 14.8% (n = 6) for LC-MS and 109 ± 12.8% (n = 4) for TOX.IS (Table 2). The ion suppression was studied by comparing standard solutions of 4-MEI with spiked samples (n = 8) at concentration levels of 50 and 100 ng/ml only for the LC-MS procedure and determined to be 7.7% at 50 ng/ml and 11.2% at 100 ng/ml (Table 2).

4.1.3. Accuracy and inter-day precision

The mean % accuracy was determined from various levels (100, 250, 500, 1000 ng/ml for TOX.IS) and (10, 25, 50, 100, 250 and 500 ng/ml for LC-MS) and was found to be 92.0 ± 13.21 (n = 4) and 106.2 ± 10.2 (n = 5) for LC-MS. The inter-day precision (expressed as %RSD) was evaluated at the same concentrations and was found to be 16.8 ± 7.4% (n = 4) for TOX.IS and 15.4 ± 2.1% (n = 5) for LC-MS (Table 2).
4.2. 4-MEI monitoring

4.2.1. TOX.IS

Eighteen (18) products, which included 13 cola type soft drinks, 2 energy drinks, 2 beers and 1 vinegar were analyzed by the TOX.IS system. The cola drinks were found to contain 4-MEI at a concentration range from 36.9 to 190.0 ng/ml. The beers and energy drinks had no detectable amounts of 4-MEI, while the level of 4-MEI in the vinegar sample was 3025.3 ng/ml.

4.2.2. LC-MS

Thirty-four (34) products, which included 15 soft drinks (14 cola type, 1 no cola type), 7 energy drinks, 2 beers, 6 vinegars and 4 sauces were analyzed with the LC-MS system. The beer samples and the no cola type soft drink contained no detectable amounts of 4-MEI. In the cola samples 4-MEI was detected at with levels ranging from 15.8 to 477.0 ng/ml (mean 224.5 ± 174.6 ng/ml). The majority of the energy drinks (57.1%) provided detectable levels with a mean value of 14.6 ± 8.8 ng/ml (6.6–22.5 ng/ml). Four of the six vinegars were found to contain 4-MEI (mean 874.8 ± 1448.6 ng/ml) with an extensive range of detected levels from 9.7 to 3034.7 ng/ml. In only one sauce sample 4-MEI was detected (17.5 ng/ml) (Table 3).

5. Discussion

Although, both analytical systems provided low inter day precision values (<16.8%) and high accuracy of the determination of 4-MEI (>92.0%), the LOD and LOQ values for the LC-MS system were much lower than those of TOX.IS. In Fig. 3, a comparison is presented of the detected 4-MEI levels in selected samples. A total of 18 samples were analyzed both with TOX.IS and LC-MS systems. Two were found negative by both analytical techniques, two samples were found negative with TOX.IS and positive with LC-MS with detected levels of 7.3 and 22.5 ng/ml (levels lower than the LOQ value of the TOX.IS). For the rest of the samples, the two techniques provided comparative detected values (r = 0.984, p < 0.001 and rs = 0.723, p = 0.003) (Fig. 3). Additionally, the intraclass correlation coefficient (ICC) showed a value of 0.879 (0.624–0.961).

Based on the labeling information, the examined samples were categorized into four sub groups according to the type of caramel which had been added (no caramel, E150, E150c and E150d). All the samples (n = 8) with no caramel color in their content, provided negative results for 4-MEI. The mean detected levels of 7.3 and 22.5 ng/ml (levels lower than the LOQ (4.0 ng/ml) of TOX.IS). E150c and E150d were found negative with TOX.IS and positive with LC-MS with detected levels of 25.0 and 100 ng/ml respectively.

Statistical differences (p < 0.001) were observed between the samples when categorized according to their color. The mean concentrations of 4-MEI in colorless/transparent samples was 14.6 ± 8.8 ng/ml in comparison to colored (brown, dark brown) samples (350.5 ± 6718.8 ng/ml) as shown in Table 3.

It is interesting that higher levels of 4-MEI were detected in samples in glass packaging (703.3 ± 1311.8 ng/ml, n = 8) followed by samples in plastic packaging (2119.1 ± 1750.4 ng/ml, n = 16) and samples in metal packaging (89.4 ± 1674.7 ng/ml, n = 10) (Table 3).

Schlee et al. (2013) reported that the average consumption of soft drinks is 224 g/day in men and 505 g/day in teenagers (14–18 years old). Using the same exposure assumptions, and based on the results presented in the present study for soft drinks, a rough estimation of the maximum daily 4-MEI intake for an adult in Greece is around 107 µg/day with a mean daily intake of 50.3 µg/day (according to the mean detected concentrations of 4-MEI in soft drinks, Table 3), while for a Greek teenager the maximum daily intake is 241 µg/day with a mean daily intake of 113 µg/day. Our
investigating the levels of 4-MEI not only in soft drinks but also in vinegars and sauces in the Greek region. Moreover, a comparison of two analytical techniques was performed without any extraction of the sample. Both TOX.IS and LC-MS systems were found to be accurate and reliable techniques for the determination of 4-MEI in the examined samples. Differences in the detected levels of 4-MEI were observed according to the labeling information (type of caramel used) or according to the packaging of the food product.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.03.028.

References


Table 3

Concentration levels of 4-MEI in the examined samples and the observed differences according the used caramel type or type of packaging.

<table>
<thead>
<tr>
<th></th>
<th>% samples with detectable 4-MEI levels</th>
<th>Mean ± SD (ng/ml)</th>
<th>Median (ng/ml)</th>
<th>Range (ng/ml)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beers (n = 2)</strong></td>
<td>0 (0%)</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Energy drinks (n = 7)</td>
<td>4 (57.1%)</td>
<td>14.6 ± 8.8</td>
<td>14.7</td>
<td>6.6–225</td>
<td></td>
</tr>
<tr>
<td>Soft drinks (n = 15)</td>
<td>14 (93.3%)</td>
<td>224.5 ± 174.6</td>
<td>216.6</td>
<td>15.8–477.0</td>
<td></td>
</tr>
<tr>
<td>Vinegars (n = 6)</td>
<td>4 (66.7%)</td>
<td>874.8 ± 1448.6</td>
<td>227.4</td>
<td>9.7–3034.7</td>
<td></td>
</tr>
<tr>
<td>Sauces (n = 4)</td>
<td>1 (25%)</td>
<td>17.5</td>
<td>--</td>
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</tr>
</tbody>
</table>

| **Caramel type**     |                                        |                   |                |               |            |
| No caramel (n = 8)   | 0 (0%)                                 | 0 ± 0.0           | --             | --            | --         |
| E150c (n = 8)        | 6 (75.0%)                              | 518.4 ± 1232.7    | 19.8           | 6.6–3034.7    |            |
| E150d (n = 13)       | 13 (100.0%)                            | 165.2 ± 161.4     | 80.1           | 9.7–472.5     |            |
| **Color**            |                                        |                   |                |               |            |
| Colorless (n = 9)    | 4 (44.4%)                              | 14.6 ± 8.8        | 14.7           | 6.6–22.5      | <0.001     |
| Brown/dark brown (n = 25) | 19 (76.0%)                      | 350.5 ± 671.8     | 190.4          | 9.7–3034.7    |            |

| **Type of packaging**|                                        |                   |                |               |            |
| Plastic (n = 16)     | 13 (81.2%)                             | 211.9 ± 175.0     | 190.4          | 15.8–477.0    | 0.158      |
| Glass (n = 8)        | 5 (62.5%)                              | 703.3 ± 1311.8    | 80.1           | 9.7–3034.7    |            |
| Metal (n = 10)       | 5 (50.0%)                              | 89.4 ± 167.4      | 22.2           | 6.6–388.6     |            |

a p-values of non-parametric tests (Mann-Whitney or Kruskal-Wallis).

Fig. 3. Correlation of detected concentration levels of 4-MEI in examined samples between LC-MS and TOX.IS.


