Determination of 4(5)-Methylimidazole in Sugar-Amino acid Aqueous Model Systems by UPLC-Q-ToF-MS

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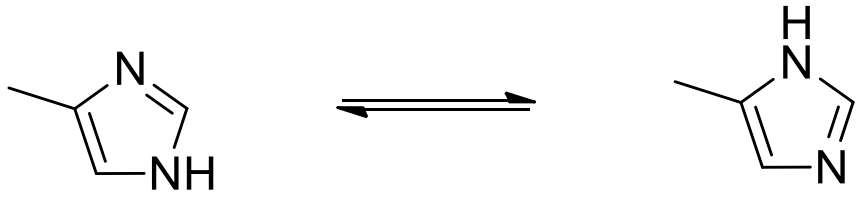
4(5)-Methylimidazole (4(5)MEI)

- **4(5)MEI** has been classified from International Agency for Research on Cancer (IARC) as potentially carcinogenic to humans.
- It is formed in food matrices during their thermal processing as a result of the Maillard reaction and is also produced during the preparation of ammonia caramel colorant additives, by the caramelization procedure.
- Early research reports have led to the hypothesis that **4(5)MEI** can be produced from the reaction between ammonia and α-dicarbonyl compounds.
- Studies employing amino acids in Maillard model systems for the investigation of **4(5)MEI** formation are still scarce.

- IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. 4-Methylimidazole. *IARC Monogr Eval Carcinog Risks Hum* **2003**, *101*, 447–459.
- Revelou et al. A Review of the Analytical Methods for the Determination of 4(5)-Methylimidazole in Food Matrices. *Chemosensors* **2021**, *9*, 322.

4(5)-Methylimidazole (4(5)MEI)

- Amino acids could be a source of nitrogen-containing compounds in foods from the Strecker degradation, and exist in significant amount in honey.
- The possibility of honey adulteration with caramel color raises concerns about the existence of **4(5)MEI** under specific conditions, apart from the addition of caramel colorants.



4-Methylimidazole 5-Methylimidazole

Figure 1. Tautomeric equilibrium of 4(5)-methylimidazole.

- **4(5)-MEI** exists in a tautomeric equilibrium in a neutral-to-basic pH aqueous solution.

- Hermosín et al. Free Amino Acid Composition and Botanical Origin of Honey. *Food Chemistry* **2003**, *83*, 263–268.
- Zábrodská & Vorlová. Adulteration of Honey and Available Methods for Detection – a Review. *Acta Vet. Brno* **2015**, *83*, 85–102.

4(5)-Methylimidazole (4(5)MEI)

- It is a highly polar molecule, characterized by the absence of chromophores, hence, liquid chromatography-mass spectrometry methods (LC-MS/MS) have been mainly applied for its determination in foods.
- Quadrupole Time-of-Flight (Q-ToF) mass analyzer offers rapid food analysis, providing high resolution, sensitivity and selectivity.

➡ The aim of this study is the development of an Ultra-high Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-Q-ToF-MS) method in order to determine **4(5)MEI** in sugar/amino acid model systems, after thermal processing.

- Revelou et al. A Review of the Analytical Methods for the Determination of 4(5)-Methylimidazole in Food Matrices. *Chemosensors* **2021**, *9*, 322.

Materials

- ❖4(5)-Methylimidazole
- ❖Glucose
- ❖Fructose
- ❖Proline
- ❖Phenylalanine
- ❖Tyrosine
- ❖Lysine

- ❖ Ammonium acetate
- ❖ Formic acid
- ❖ Hydrochloric acid
- ❖ Ammonium hydroxide
- ❖ Methanol (HPLC grade)
- ❖ Acetonitrile (ACN) (LC-MS grade)
- ❖ Ultra high purity water
- ❖ Solid Phase Extraction (SPE) cartridges (Bond Elut SCX, 500 mg, Agilent Technologies)

Standard solutions

Stock solution (1000 mg L^{-1}) of **4(5)MEI** was prepared using ACN and stored in dark glass vial at −20 °C. Calibration curve of 4(5)MEI was constructed using the standard concentrations of 10.0, 8.0, 5.0, 3.0, 1.0, 0.8, 0.5, 0.3, 0.1 mg L^{-1} via dilution with ACN.

Methods

Preparation of Aqueous Model Systems

- Eight model systems were prepared (Table 1) by mixing the appropriate amount of sugar (glucose, fructose) and amino acid (proline, phenylalanine, tyrosine, lysine).

- Water was added in order to reach the quantity of 100 g for each model system.

- The aqueous model systems were heated at 100 °C for 60 h in a Tv10b heating oven (Memmert, Germany).

Methods

SPE Procedure

- 10 g of sample were weighted into a glass vial and diluted with 10 mL of water.
- The solution was acidified with 20 μL of 0.1 M HCl.
- SCX cartridges were activated with 2 mL of methanol and 2 mL of 1% (v/v) formic acid solution.
- The sample solution was loaded into the column under vacuum and impurities were washed out with 4 mL of methanol and 4 mL of 1% (v/v) formic acid solution.
- Elution of **4(5)MEI** was achieved using 5 mL of 5% (v/v) methanolic ammonia solution.
- The solvent was remove under N_2 gas flow stream and the residue was dissolved with 1 mL of 10% (v/v) aqueous ACN.

Methods

UPLC-Q-ToF-MS

- The high resolution mass spectrometry spectra were recorded on an Agilent 6530 Quadrupole Time of Flight LC-MS system (Q-ToF-MS), with an ESI source, coupled with Agilent 1290 Infinity UPLC system and an autosampler.

- Nitrogen was used as the collision gas. Positive and negative electrospray ionization (ESI) was used for the MS experiments.

- The data acquisition was carried out with Agilent MassHunter software.

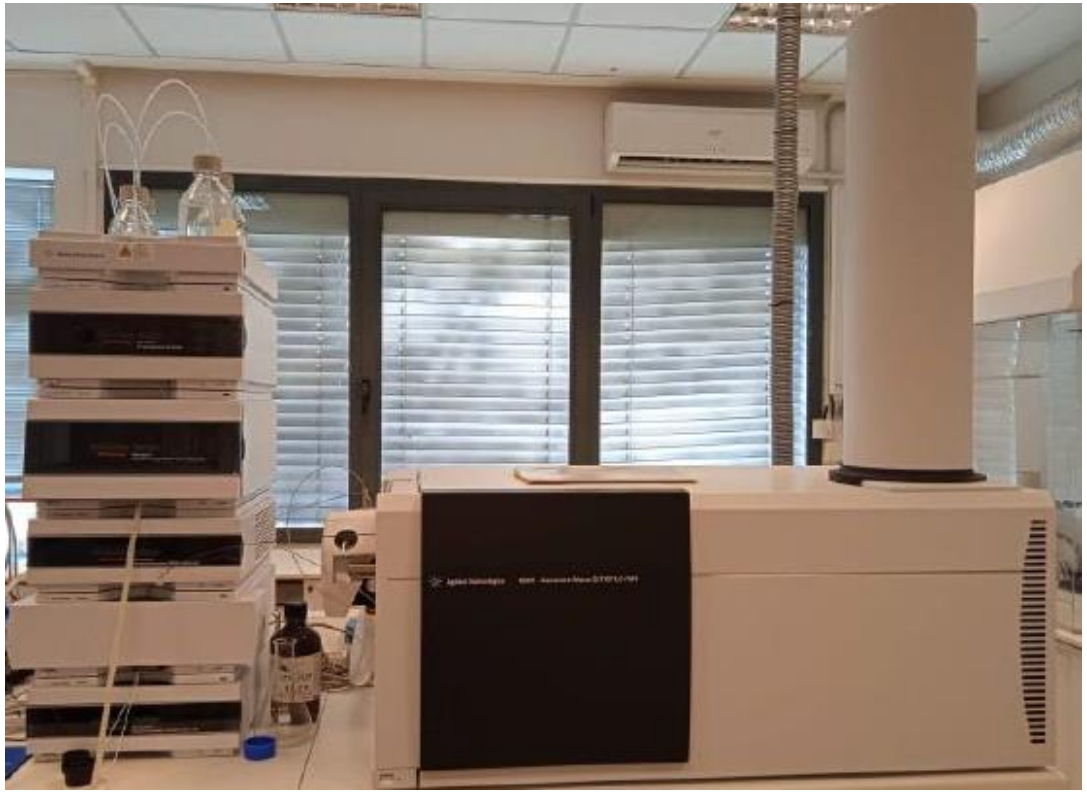


Figure 2. Agilent 6530 LC-Q-ToF-MS

Methods

Q-TOF-MS conditions

- Drying gas, 12 L/min
- Gas temperature, 350 ˚C
- Fragmentor, 100V
- Skimmer, 65 V
- Capillary voltage, 3000 V
- Nebulizer gas, 50 psi
- Acquisition rate, 1 spectra/s (threshold 200 Abs, 0.01% rel.)
- MS scan range, 50-1500

Methods

Chromatographic Study

- Performed with a NUCLEOSHELL Bluebird (RP 18 EC, 2.7 µm particle size, 100 mm length, 4.6 mm i.d.) (Macherey-Nagel, Düren, Germany) column.
- Mobile phase: A = water/ammonium acetate 5 mM; mobile phase B = ACN.
- Gradient: 0 min 4% B, 5 min 40% B, 10 min 100% B, 17 min 4% B, 25 min 4% B.
- Total run time including column equilibration: 25 min.
- Injection volume: 5 µL.
- Flow rate: 1.0 mL/min.
- Column oven temperature: 40 °C.

Results and Discussion

UPLC-Q-ToF-MS Method

- **4(5)-MEI** was studied in positive and negative ESI mode at different fragmentor (100 V, 120 V, 150 V) and capillary voltage conditions (3000 V, 4000 V).
- In negative ESI mode the $[M-H]^-$ ion was not detected.
- In positive ESI mode, the optimum abundance of the $[M+H]^+$ ion was observed at fragmentor 100 V and capillary voltage 3000 V.
- The $[M+Na]^+$ ion was not observed.
- **4(5)-MEI** was detected at retention time 1.72 min at m/z 83.0606 (Δ 2.41 ppm).

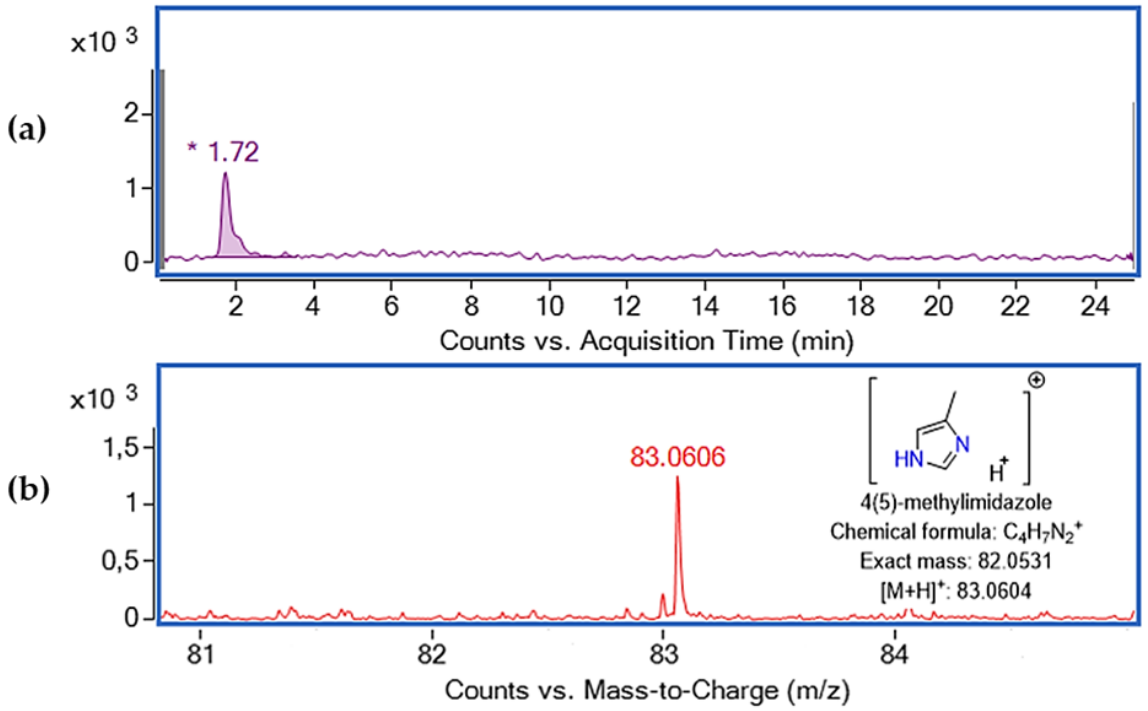


Figure 3. (a) Extracted ion chromatogram of 1 mg L^{-1} standard solution and (b) mass spectrum of 1 mg L^{-1} standard solution.

Results and Discussion

UPLC-Q-ToF-MS Method

- The peak area of the extracted ion chromatograms was utilized for the quantification of 4(5)MEI.

- The linearity of the UPLC-Q-ToF-MS method was determined by the construction of a calibration curve at different concentrations.

- Limit of detection (LOD) and quantification (LOQ) were 1.7 mg L^{-1} and 5.1 mg L^{-1}, respectively, while linearity was R^2=0.9908.

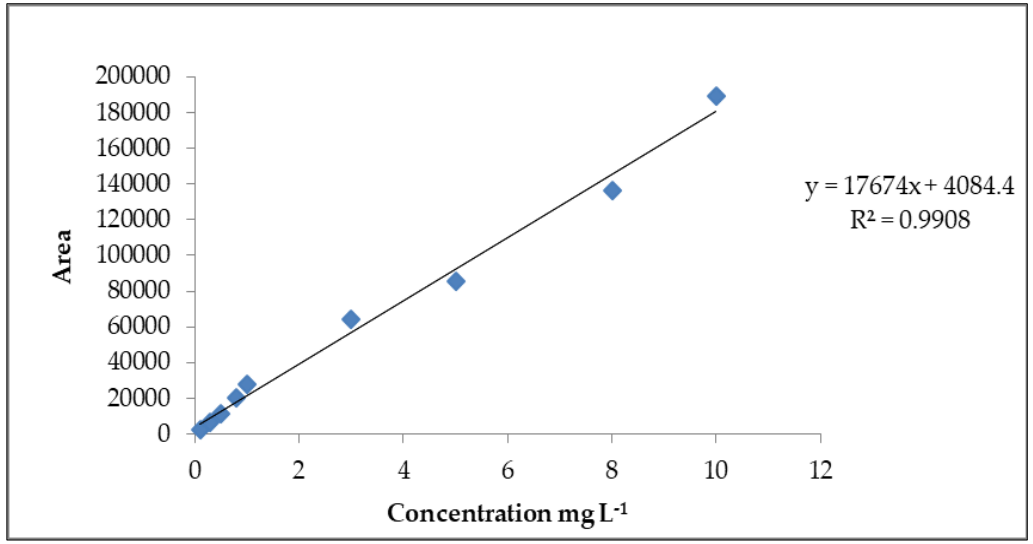


Figure 4. Calibration curve of 4(5)MEI.

Results and Discussion

Analysis of Aqueous Model Systems

Table 1. Aqueous model systems of sugars-amino acids analyzed by UPLC-Q-ToF-MS and the concentration of 4(5)MEI after their thermal treatment.

Sample	Model system	Concentration (μg mL^{-1})
1	60 g glucose + 100 mg proline	1.1
2	60 g glucose + 20 mg phenylalanine	0.4
3	60 g glucose + 10 mg tyrosine	0.2
4	60 g glucose + 10 mg lysine	1.3
5	70 g fructose + 100 mg proline	3.5
6	70 g fructose + 20 mg phenylalanine	2.5
7	70 g fructose + 10 mg tyrosine	3.0
8	70 g fructose + 10 mg lysine	0.9

Results and Discussion

Analysis of Aqueous Model Systems

- 4(5)-Methylimidazole was detected in all model systems.
- The combination of fructose and proline provided the highest concentration of 4(5)MEI.
- The lowest concentration was detected in the glucose–tyrosine system (0.2 μg mL^{-1}), however, in the fructose–tyrosine system the concentration of 4(5)MEI was significantly elevated (3.0 μg mL^{-1}).
- In all model systems of amino acids with fructose the concentration of 4(5)MEI was increased, except for fructose-lysine where it was lower (0.9 μg mL^{-1}), compared to glucose-lysine (1.3 μg mL^{-1}).

The results indicate that the formation of 4(5)MEI is differently affected from each amino acid.

Results and Discussion

Analysis of Aqueous Model Systems

❖ In the current study the amount of fructose was increased by 10 g in relation to glucose.

The results indicate that formation of 4(5)MEI may be affected by the amount of sugar.

❖ These results are in agreement with a research study on glucose-ammonia systems, heated at 150°C for 2 h, where the authors observed that the concentration of 4(5)MEI increases with increasing glucose concentration.

❖ The amino acids and sugars used in this study are main components of honey.

Since 4(5)MEI was detected in the model systems of the current study, future research should focus on the conditions of possible formation of 4(5)MEI in honey.

- Jang et al. Formation of 4(5)-Methylimidazole and Its Precursors, α-Dicarbonyl Compounds, in Maillard Model Systems. *J. Agric. Food Chem.* **2013**, *61*, 6865–6872.

Conclusions

- An accurate analytical method for the determination of 4(5)MEI was developed employing UPLC-Q-ToF-MS at positive ESI mode.
- Eight aqueous model systems were prepared with sugar (glucose, fructose) and amino acid (proline, phenylalanine, tyrosine, lysine), which are main components of honey.
- The model systems were heated at 100 °C for 60 hours, in order to study the formation of 4(5)MEI.
- The results indicate that the formation of 4(5)MEI is differently affected from each amino acid as well as the amount of sugar.
- The proposed UPLC-Q-ToF-MS method can be used for future applications in other food matrices.
- Further research is required to examine the possibility of 4(5)MEI formation in honey.

Thank you for your attention