Simultaneous Quantitation of 2- and 4-Methylimidazole in Food Products with Monolithic Type Sample Preparation Approach and LC-MS/MS

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Introduction

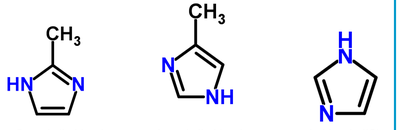
Caramel coloring is a dark brown liquid or solid material used as a color additive. Caramel coloring results from the controlled heat treatment, often under pressure, of various food-grade carbohydrates, such as high-dextrose corn syrup. There are four caramel color classes (I, II, III and IV). Caramel colors III and IV are manufactured using processes that expose carbohydrates to ammonia (with or without sulfites). These manufacturing processes exploit the reaction pathways that the Maillard reaction can follow to result in the characteristic caramel color.

Exposing carbohydrates in the presence of ammonia to create caramel color via the Maillard reaction can result in byproducts that are potentially toxic or harmful. One of those byproducts is 4-methylimidazole (4-MEI), a compound that is classified as a potential cancer-causing agent (1). A structural isomer of 4-MEI, 2-methylimidazole (2-MEI), is also commonly believed to be a potential byproduct, although 2-MEI is not in caramel color (2). A method that covers both 2- and 4-MEI allows for a single sample prep and analysis method so that companies can demonstrate concentrations of both.

The work presented here covers a sample preparation and analytical methodology useful for identification and quantification of 4-MEI, along with 2-MEI, in caramel colors III and IV and in beverages and foods containing caramel color III and IV. Using monolithic solid phase extraction (SPE) along with liquid chromatography and tandem mass spectrometry (LC-MS/MS), excellent linearity and sensitivity can be achieved.

Experimental

2-MEI and 4-MEI are structural isomers that are basic and very hydrophilic. These chemical properties can be taken advantage of when developing and refining the sample preparation method.



2-methylimidazole	4-methylimidazole	Imidazole (IS)
MF: C4H6N2	MF: C4H6N2	MF: C3H4N2
MW: 82	MW: 82	MW: 68
Log P: 0.031	Log P: 0.341	Log P: -0.099
pKa: 7.86	pKa: 7.51	pKa: 7.0

Structures and Properties for 2-MEI, 4-MEI and Imidazole (IS)

Experimental, cont.

SPEC Sample Preparation

Agilent SPEC SPE discs are made of a monolith-like material, where the functional group is embedded onto a substrate, resulting in an SPE cartridge with no loose sorbent. This delivers consistent flow, with no channeling and makes it amenable to extracting a range of sample types.

Solid Phase Extraction

- All samples - solid, liquid or powder – need to be pre-prepped prior to solid phase extraction
  - Mix 2 g of sample with 10 mL methanol with 1% formic acid (FA) and shake 3 min
  - Centrifuge 10 min at 5000 rpm
  - Transfer 5 mL of supernatant to a new tube and evaporate to dryness
  - Reconstitute in 2 mL water with 2% FA
- Sample extraction using SPEC MP1, 30 mg/disk (mixed phase nonpolar/strong cation exchanger)
  - Condition Columns: Methanol, then water with 2% FA, 1 mL each
  - Load: Reconstituted sample
  - Wash: Water (2% FA), then methanol, 1 mL each; dry columns under vacuum at 12-13" Hg for 3 min
  - Elute: Ammonium hydroxide/Methanol (1:9) 1 mL x 2
  - Dry samples and reconstitute in 1 mL water with 0.1% FA
- Analyze samples by LC-MS/MS

LC-MS/MS Parameters

An Agilent LC-triple quadrupole mass spectrometer was used for this analysis. An Agilent Pursuit 3 PFP LC column was selected to provide a rapid analysis, with retention times less than 2 minutes using an isocratic method.

Agilent 1200 LC Parameters

- Column: Pursuit 3 PFP, 2.0 x 50 mm, 3 µm
- A: Water (0.1% FA)
- B: Methanol (0.1% FA)
  Isocratic: 99% A:1% B
- Flow rate: 0.2 mL/min
- Column Temp: 10 °C

Agilent 6460 Triple Quadrupole MS Settings

- MS Conditions: Gas Temp: 300 °C
  Gas Flow: 10 L/min
  Nebulizer: 40 psi
  Sheath Gas Temp: 350 °C
  Sheath Gas Flow: 12 L/min
  Capillary Voltage: 3500 V
  Nozzle Voltage: 500 V

Results

Table 1. MS Acquisition Parameters

Compounds	Transitions (m/z)	Fragmentor (V)	CE (V)
2-methylimidazole (2-MEI)	83.1 > 42.1 83.1 > 81.9	57 57	19 3
4-methylimidazole (4-MEI)	83.1 > 56.0 83.1 > 42.1	47 47	15 27
Imidazole (IS)	69.1 > 42.1 69.1 > 67.8	57 57	19 7

The mass spectrometer acquisition parameters, including transitions and precursor/product ions were optimized for each compound (Table 1). All compounds were acquired in a single acquisition group, with retention times and a total run time less than 2 minutes.

Using the Agilent Pursuit 3 PFP analytical column and pre-spiked samples, the chromatography was optimized, using an isocratic method. As shown in Figure 1, peak shapes and resolution were sufficient for a sample that was spiked before extraction with 200 ppb of 4-MEI and 2-MEI plus the internal standard.
The method also demonstrated good linearity, with replicate injections at 10, 50, 100, 200 and 400 ppb (Figure 2). Six replicate injections were performed at each level.
Based on this curve, relative recovery and precision were assessed at 100, 200 and 400 ppb (Table 2). These relative recoveries used samples that were spiked before extraction, and the values were calculated based on the calculated amount relative to the expected concentration at each level.

Figure 1. Pre-Spiked Sample at 200 ppb

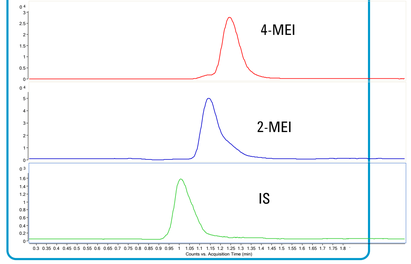
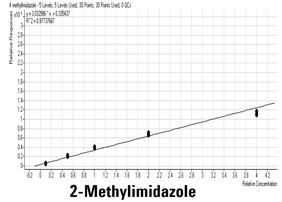
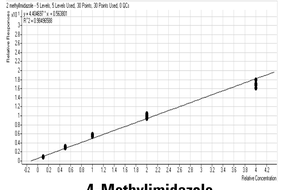


Figure 2. Calibration Curves for 2-MEI and 4-MEI



2-Methylimidazole



4-Methylimidazole

Table 2. Relative Recoveries and RSDs for Pre-Spiked QC Samples

Compounds	100 ppb		200 ppb		400 ppb	
	Rel Recovery	%RSD	Rel Recovery	%RSD	Rel Recovery	%RSD
2-methylimidazole (2-MEI)	103%	7.7%	102%	1.0%	100%	7.1%
4-methylimidazole (4-MEI)	116%	9.5%	125%	4.3%	117%	7.0%

Results, cont.

The optimized sample preparation and analysis methods were used to analyze incurred samples of pumpernickel bread, which listed caramel color as one of its ingredients. The results of this analysis, using 2 g of bread, are shown in Figure 3. 4-MEI was found in this bread at less than 10 ppb (calculated amount of 3.9 ppb).

Figure 3. Incurred Sample: Pumpernickel Bread

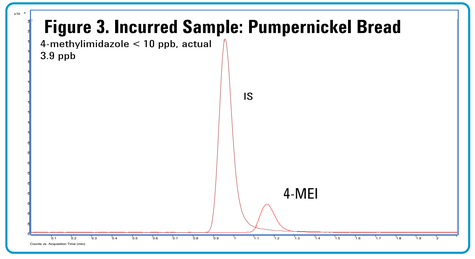
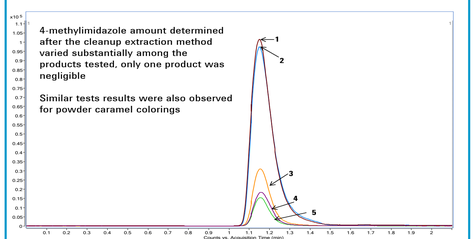


Figure 4. Series of Liquid Caramel Colorings: 4-MEI Determination



Five different liquid caramel color formulations were extracted and analyzed according to the described method. The concentration of 4-MEI detected varied greatly across these different products. (Figure 4) However, only one contained a negligible concentration of 4-MEI.

This sample prep method combined with LC-MS/MS detection and quantification was also used on powered caramel color, with similar results. In all cases, the quantity of 4-MEI detected was less than 250 ppm, which is the specification limit set by some agencies.

Conclusions

- The Agilent SPEC monolithic SPE method offers a simple sample preparation approach to determine 2/4-methylimidazoles in food and food coloring.
- Specification limit of 250 ppm for 4-MEI in caramel coloring is much higher than the amounts observed in the sample analyzed using our extraction and separation procedures.
- The SPEC material cleans the sample nicely and can be used across all food products; dry, liquid, powder.
- The Agilent Pursuit 3 PFP column allows a very quick aqueous isocratic method for the analysis, less than 2 minutes.

1) http://www.oehha.ca.gov/prop65/law/100711MEI_NSRL.html, retrieved September 7, 2012
2) http://www.iacmcolor.org/color-info/2-methylimidazole-2-mei-is-not-present-in-caramel-colors, retrieved September 6, 2012
3) Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council Text with EEA relevance. Official Journal L 083 , 22/03/2012, P. 0001 - 0295