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Simultaneous Quantitation of 2-Acetyl-4-tetrahydroxybutylimidazole, 2- and 4-Methylimidazoles, and 5-Hydroxymethylfurfural in Beverages by Ultrahigh-Performance Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT: An ultrahigh-performance liquid chromatography (UHPLC) tandem mass spectrometric (MS/MS) method was developed for the simultaneous quantification of 2-acetyl-4-tetrahydroxybutylimidazole (THI), 2- and 4-methylimidazoles (2-MI and 4-MI), and 5-hydroxymethylfurfural (HMF) in beverage samples. A C30 reversed-phase column was used in this method, providing sufficient retention and total resolution for all targeted analytes, with an MS/MS instrument operated in selected reaction monitoring (SRM) mode for sensitive and selective detection using isotope-labeled 4-methyl-d_3-imidazole (4-MI-d_3) as the internal standard (IS). This method demonstrates lower limit of quantification (LLOQ) at 1 ng/mL and coefficient of determination (r^2) >0.999 for each analyte with a calibration range established from 1 to 500 ng/mL. This method also demonstrates excellent quantification accuracy (84.6–105% at 5 ng/mL, n = 7), precision (RSD < 7% at 5 ng/mL, n = 7), and recovery (88.8–99.5% at 10, 100, and 200 ng/mL, n = 3). Seventeen carbonated beverage samples were tested (n = 2) in this study including 13 dark-colored beverage samples with different flavors and varieties and 4 light-colored beverage samples. Three target analytes were quantified in these samples with concentrations in the range from 284 to 644 ng/mL for 4-MI and from 706 to 4940 ng/mL for HMF. THI was detected in only one sample at 6.35 ng/mL.

KEYWORDS: *2-acetyl-4-tetrahydroxybutylimidazole, methylimidazole, 5-hydroxymethylfurfural, carcinogen, beverage, UHPLC, mass spectrometry*

INTRODUCTION

2-Acetyl-4-tetrahydroxybutylimidazole (THI) and 2- and 4-methylimidazoles (2-MI and 4-MI) are undesired byproducts produced during the manufacture of caramel color used to darken food products such as carbonated beverages and soy sauces. 5-Hydroxymethylfurfural (HMF) is formed during the thermal treatment of carbohydrate-containing foods and has been found in different products at various levels.[1] HMF has also been used as a quality indicator for certain foods such as honey, because its concentration increases during conditioning and storage.[2,3] Although the correlation of adverse health effects and exposure to HMF is not conclusive,[4,5] analytical measurements of HMF in foods are necessary for an objective risk assessment relating to human exposure as well as for quality evaluation for certain foods. THI was reported as an immunosuppressive compound.[6,7] Studies from the National Toxicology Program (NTP) and other researchers concluded that there is clear evidence of the carcinogenicity of 2- and 4-MI on the basis of animal studies.[8−12] In 2011, the International Agency of Research on Cancer (IARC) listed both methylimidazoles as group 2B compounds that are possibly carcinogenic to humans.[13] The Office of Environmental Health Hazard Assessment (OEHHA) in California listed 4-MI as carcinogen in January 2011[14] with a proposed no significant risk level (NSRL) at 29 μg per person per day.[15] On the other hand, the European Food Safety Authority concluded that exposure to 4-MI from caramel color was not of concern.[16] Although the health effect of exposure to methylimidazoles is controversial, it would be prudent to take precautions, especially for sensitive populations that may be susceptible to excessive exposure. Thus, a quantitative analytical measurement for the four above-mentioned compounds is desired for reliable exposure and risk assessments.

Conventional methods for quantitation of these target compounds include gas chromatography (GC) methods,[17−20] which involve labor intensive procedures such as hot solvent extraction and acetyl derivatization and are thus not amenable for high-throughput analysis. Several liquid chromatographic (LC) methods have also been reported.[20−26] However, to the authors' best knowledge, no previous study has been reported for the simultaneous quantitation of all four undesired compounds in beverages and other food products.

This study describes a high-throughput ultrahigh-performance liquid chromatography (UHPLC-MS/MS) method for the simultaneous quantitation of four compounds, THI, 2-MI, 4-MI, and HMF, in various carbonated beverage products. In this method, carbonated beverage samples were degassed by sonication, diluted, and directly analyzed. Chromatography was performed on a UHPLC system, and separation was achieved on a C30 reversed-phase column within 5 min. The MS/MS instrument was operated in selected reaction monitoring (SRM) mode for the best sensitivity and selectivity.

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Seventeen carbonated beverage samples were assayed using this method with positive quantification of THI, 4-MI, and HMF.

MATERIALS AND METHODS

Chemical and Reagents. Chemical standards used in this study were purchased from Sigma-Aldrich (2-MI, 4-MI, HMF; St Louis, MO) and Cayman Chemical (THI; Ann Arbor, MI). Isotope-labeled internal standard 4-MI-d_3 was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Methanol and acetonitrile were obtained from Honeywell Burdick & Jackson (HPLC-UV grade, Muskegon, MI). Ammonium hydroxide was purchased from Sigma-Aldrich for mobile phase preparation. Deionized (DI) water was obtained from a Millipore water station. Stock solutions of each individual standard and IS were prepared by dissolving the appropriate amount of each pure chemical in DI water to the concentration of 1 mg/mL. Working standard solutions were prepared by mixing individual stock solutions in DI water at 10 μg/mL and then diluted to 1 μg/mL and 100 ng/mL. IS stock solution was diluted in DI water to 10 μg/mL to prepare calibration standards and spike unknown samples. Calibration standards were prepared from working solutions to six levels: 1, 5, 10, 50, 100, and 500 ng/mL with IS spiked at 100 ng/mL for each level.

Sample Preparation. Bottled carbonated beverage samples were purchased from local grocery stores. All samples were stored at room temperature until opened for analysis. A 20 mL aliquot of each sample was poured into a 150 mL specimen cup and degassed for 1 min in a sonication bath at room temperature. A 100 μL aliquot of each degassed sample was then pipetted to a 1.5 mL autosampler vial and spiked with 10 μL of a 10 μg/mL IS working solution, diluted with 900 μL of deionized water (DI water), and vortex mixed, and then 10 μL of each prepared sample was injected for UHPLC-MS/MS analysis.

UHPLC-MS/MS Conditions. Chromatography was performed on an UltiMate 3000 RS UHPLC system (Thermo Scientific, Sunnyvale, CA), and retention and separation of target analytes were achieved on an Acclaim C30 reversed-phase column (2.1 × 150 mm, 3 μm particle size, Thermo Scientific, Sunnyvale, CA) with isocratic elution. The mobile phase consisted of 10% methanol, 5% ammonium hydroxide (0.7% in DI water), and 85% DI water and was delivered at a flow rate of 0.3 mL/min. The column temperature was set at 15 °C.

A Quantum TSQ Access MAX MS/MS instrument (Thermo Scientific, San Jose, CA) was selected as the detector and was operated in SRM mode to achieve the best selectivity and sensitivity. An electrospray ionization (ESI) interface with a heated ESI probe (HESI-II) was used to couple the UHPLC and MS/MS systems. The source parameters were set as follows: the spray voltage, vaporizer temperature, capillary temperature, sheath gas, and auxiliary gas were set at 4500 V, 350 °C, 200 °C, 60, and 60 arbitrary units, respectively. Two SRM transitions were used for the quantitation (Q-SRM) and confirmation (C-SRM) of each target analyte with collision energy optimized for each SRM transition. Details of the SRM transitions are listed in Table1.

Table 1. Studied Compounds and SRM Scan Events

analyte	retention time (min)	time event (min)	precursor ion (m/z)	Q-SRM (CID) (m/z) (V)	C-SRM (CID) (m/z) (V)
THI	1.80	1.6–2.1	−229	109 (20)	151 (17)
2-MI	2.45	2.1–3.8	+83	42 (24)	56 (18)
4-MI	3.12	2.1–3.8	+83	56 (18)	42 (24)
IS	3.12	2.1–3.8	+86	59 (23)	N/A[a]
HMF	4.05	3.8–5.0	+127	53 (24)	81 (18)

[a]N/A, not available.

RESULTS AND DISCUSSION

Method Development. The objective of this study was to develop a high-throughput quantitative method for simultaneous determination of all four target analytes in beverage samples. 2-MI and 4-MI are isomers with identical molecular weights. Our initial investigation on MS/MS instrumentation for SRM development showed the same fragmentation paths for both compounds, that is, the same precursor and product ions. Therefore, chromatographic separation is essential for the accurate quantitation of each individual analyte. Various columns with different selectivities were evaluated for chromatographic performance, including retention and resolution. Among the tested columns, including C8, C18 reversed-phase columns, a mixed-mode column featuring combined reversed-phase, anion exchange and cation exchange mechanisms, a HyperCarb column composed of pure porous graphitic carbon, and a C30 reversed phase column, the C30 reversed column provided the best results with sufficient retention and total resolution of all target analytes and was selected for the rest of the study. Detailed chromatographic conditions are described in the prior section. Minimum retention was observed for THI at a retention time of 1.80 min, thus ensuring the separation of THI from early-eluting impurities that may cause ion suppression and/or interferences with MS detection. Although a minimum retention factor of >2 would be preferred, the retention for THI was sufficient under these conditions for this particular analysis based on later assays of real samples: the peak shape was well maintained, and the deviation of retention time was <0.1 min among assays of calibration standards, real samples, and recovery samples during an 8 day period. The optimized SRM chromatograms of a blank carbonated beverage sample spiked with 10 ng/mL of each target analyte are shown in Figure 1. The divert valve on the TSQ instrument was activated before the elution of the first target analyte when the LC eluent was directed to waste to avoid possible MS source contamination.

The choice of organic solvent appeared to be critical for the quantitation of 2-MI and 4-MI. Although total chromatographic separation can be achieved with either methanol or acetonitrile, strong interference for both methylimidazoles with MS detection was observed when acetonitrile was used as the mobile phase organic modifier. The interference can be explained by the acetonitrile solvent cluster ion $[2M + H]^+$, which has the same mass-to-charge ratio (m/z) at 83 as the SRM precursor ions. Therefore, methanol was used instead in this study. THI showed a strong deprotonated molecular ion $[M - H]^-$ at m/z 229, and the other analytes exhibited strong protonated molecular ions $[M + H]^+$: 2- and 4-MI at m/z 83, and HMF at m/z 127, respectively. The observed protonated/deprotonated molecular ions were used as precursor ions, and SRM transitions were determined by infusing standard compounds and product ions optimized using the instrument tuning program. The two most intensive fragment ions were selected as product ions for quantitation (Q-SRM) and confirmation (C-SRM) as shown in Table 1. Although two identical SRM transitions (m/z 83→42 and 83→56) were observed for 2-MI and 4-MI, significant difference was observed for their relative intensities. As seen in Figure 1, for 2-MI, a relatively stronger response was observed with SRM 83→42, which was used as Q-SRM. However, 4-MI demonstrated the opposite relative intensity; thus, SRM 83→56 was used as the Q-SRM for 4-MI. The difference in relative intensity of the two SRM transitions (Q-SRM and C-SRM) can be monitored, and the ratio could be used as additional confirmation supplementing chromatographic retention time. A total of nine SRM transitions were arranged into three timed segments to reduce

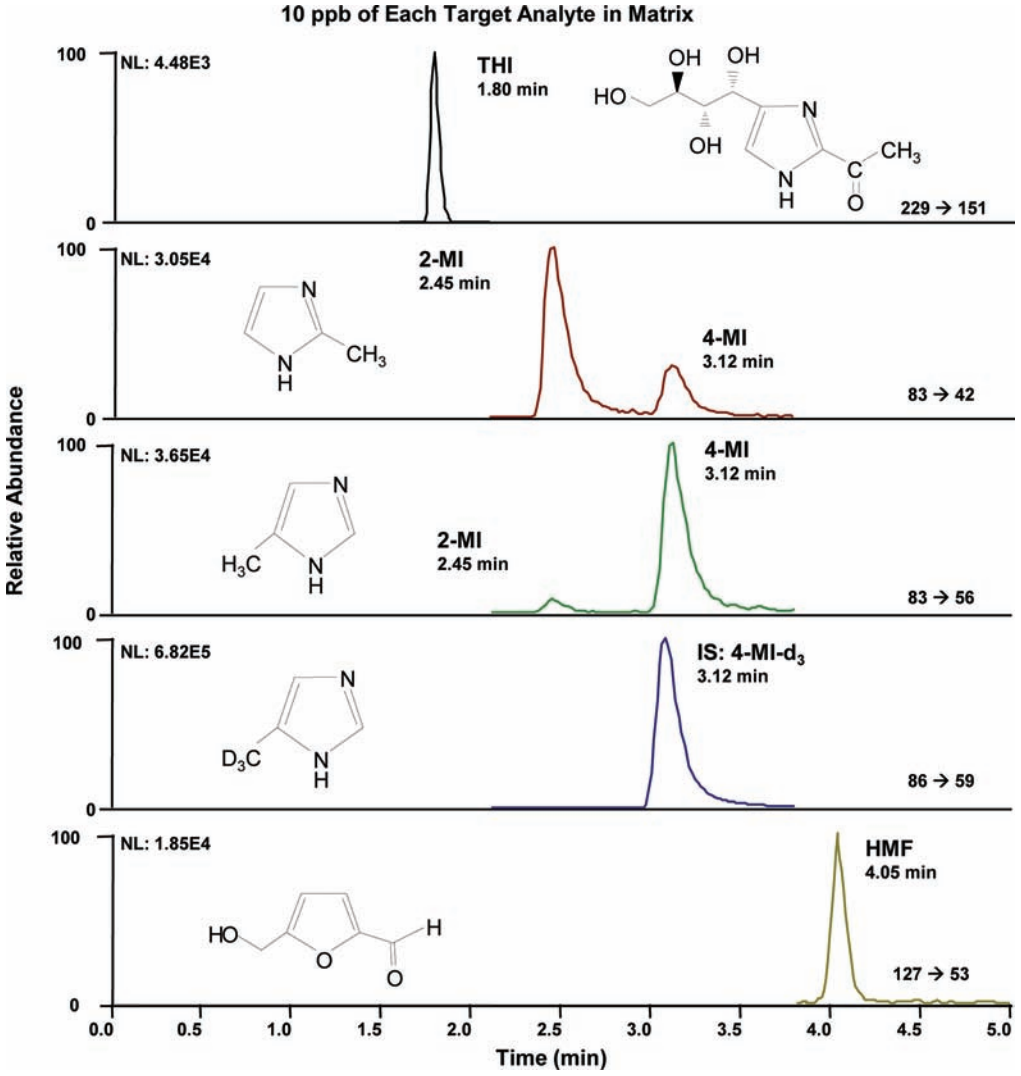

Figure 1. THI, HMF, 2-MI, and 4-MI spiked in a blank carbonated beverage sample.

total analytical cycle time at any specific retention time, thus consequently maximizing the number of data points gathered across the chromatographic peaks improving quantitative accuracy.

Method Performance. The method performance was evaluated against quality parameters such as specificity, carry-over, linearity and calibration, correlation of determination (r^2), detection limit, precision, accuracy, and recovery.

Method specificity was confirmed by the absence of quantifiable peaks with the assay of blank samples and positive peak detection at specific retention times with the injection of each individual standard. Carry-over was evaluated by injecting two blanks immediately after the assay of a standard with the highest concentration in the calibration range at 500 ng/mL. No quantifiable peaks were observed in the subsequently injected blank samples, indicating the absence of system carry-over. Calibration curves were generated from calibration standards from LLOQ to 500 ng/mL. LLOQ was determined as the lowest concentration in calibration standards with observed Q-SRM signal-to-noise (S/N) >10 and C-SRM S/N >3. In this study, the LLOQ was observed at 1 ng/mL for all analytes. A second approach was also employed to statistically evaluate the instrument detection limit. The method detection limit (MDL) was calculated following the equation MDL = $S \times t_{(99\%,\ n=7)}$, where S is the standard deviation of a series of replicate assays near the detection limit, and t is Student's t at a 99% confidence interval. The calculated MDLs ranged from 0.81 to 0.89 ng/mL and were comparable to the prior determined LLOQs. In the generation of calibration curves, a linear fit was used to fit experimental data, and $1/x$ weighting was used for each analyte to achieve better quantitation accuracy at lower levels. Excellent linearity was achieved with a correlation of determination (r^2) observed >0.999 for each analyte as shown in Table 2. The precision and accuracy were evaluated by seven replicate assays of standard at 5 ng/mL. The

Table 2. Linearity, Calibration,[a] Precision, Accuracy, and Detection Limit

		5 (ng/mL) (n = 7)			
analyte	r^2	% accuracy	% RSD	MDL[b] (ng/mL)	LLOQ (ng/mL)
THI	0.9991	84.6	6.71	0.89	1
2-MI	0.9994	86.0	6.25	0.85	1
4-MI	0.9996	92.0	5.57	0.81	1
HMF	0.9996	105	5.10	0.84	1

[a]Calibration range: 1−500 ng/mL. [b]MDL, method detection limit.

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precision and accuracy were addressed by %RSD and %accuracy (calculated by observed amount/specified amount × 100%), respectively. As seen in Table 2, excellent precision was observed for all analytes with %RSD <7% for performed experiments; %accuracy was observed from 84.6% (THI) to 105% (HMF), indicating accurate measurements could be achieved using this method. Recovery was evaluated by spiking target analytes at three concentrations (10, 100, and 200 ng/mL, n = 3) in a blank matrix (sample 14), which was found to contain no quantifiable target analytes when assayed. The recovery was calculated by %recovery = quantified amount/spiked amount × 100%. The results are shown in Table 3. Observed recoveries ranged from 88.8% (THI, 10 ng/mL) to 99.5% (4-MI, 100 ng/mL).

Table 3. Recovery of Target Analytes[a]

	10 (ng/mL)	100 (ng/mL)	200 (ng/mL)
THI	88.8 (4.23)	88.9 (0.73)	91.9 (2.72)
2-MI	94.1 (3.74)	91.1 (3.70)	91.9 (1.22)
4-MI	96.0 (2.22)	99.5 (2.80)	98.0 (2.29)
HMF	93.5 (2.63)	95.9 (2.93)	93.6 (3.48)

[a] n = 3; results shown are % recovery with %RSD in parentheses.

Analysis of Carbonated Beverages. As mentioned under Sample Preparation, 17 carbonated beverage samples were prepared following described procedures and analyzed for target analytes. Use of different dilution factors and reanalysis may be necessary for samples with observed concentrations beyond the calibration range. The results are shown in Table 4.

Table 4. Quantified THI, 4-MI, and HMF in Tested Samples[a]

sample	color	THI	4-MI	HMF
1	dark	–[b]	398	2820
2	dark	–	319	2312
3	dark	–	284	2010
4	dark	–	391	2894
5	dark	–	416	770
6	dark	–	389	706
7	dark	–	412	4940
8	dark	–	411	1760
9	dark	6.35	299	1300
10	dark	–	692	2320
11	dark	–	644	1600
12	dark	–	568	2320
13	light	–	–	4490
14	light	–	–	–
15	light	–	–	1530
16	light	–	–	2270
17	dark	–	356	2460

[a] Results are in the units of (ng/mL) in original sample. Each sample was measured in duplicate (n = 2). [b] –, not detected.

No 2-MI was detected in any of the tested samples; thus, 2-MI is not included in the results table. THI was found only in sample 9, which is a root beer sample. 4-MI was observed in all dark-colored samples with concentrations from 284 to 692 ng/mL and absent in light-colored samples (sample 13–16), indicating the main source of 4-MI was the caramel color as shown on the contents label. HMF was detected in most tested samples, except for sample 14, in a higher concentration range from 706 ng/mL to 4.94 μg/mL.

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REFERENCES

(1) Bachmann, S.; Meier, M.; Kanzig, A. 5-Hydroxymethyl-2-furfural (HMF) in Lebensmitteln. *Lebensmit-telchemie* **1997**, *51*, 49–50.

(2) Fallico, B.; Arena, E.; Zappala, M. Roasting of hazelnuts. Role of oil in colour development and hydroxymethylfurfural formation. *Food Chem.* **2003**, *81*, 569–573.

(3) Zappalà, M.; Fallico, B.; Arena, E.; Verzera, A. Methods for the determination of HMF in honey: a comparison. *Food Control* **2005**, *16*, 273–277.

(4) Abraham, K.; Gürtler, R.; Berg, K.; Heinemeyer, G.; Lampen, A.; Appel, K. E. Toxicology and risk assessment of 5-hydroxymethylfurfural in food. *Mol. Nutr. Food Res.* **2011**, *55*, 667–678.

(5) Janzowski, C.; Glaab, V.; Samimi, E.; Schlatter, J.; Eisenbrand, G. 5-Hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. *Food Chem. Toxicol.* **2000**, *38*, 801–809.

(6) Gugasyan, R.; Losinno, C.; Mandel, T. The effect of 2-acetyl-4-tetrahydroxybutylimidazole on lymphocyte subsets during a contact hypersensitivity response in the NOD mouse. *Immunol. Lett.* **1995**, *46*, 221–227.

(7) Houben, G. F.; Berg, H. v. d.; Kuijpers, M. H. M.; Lam, B. W.; Loveren, H. v.; Seinen, W.; Penninks, A. Effects of the color additive caramel color III and 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) on the immune system of rats. *Toxicol. Appl. Pharmacol.* **1992**, *113*, 43–54.

(8) National Toxicology Program. *Toxicity and Carcinogenesis Studies of 2-Methylimidazole in F344/N Rats and B6C3F1 Mice*; Public Health Service, National Institute of Health: Research Triangle Park, NC, 2004.

(9) National Toxicology Program. *Toxicology and Carcinogenesis Studies of 4-Methylimidazole in F344/N Rats and B6C3F1 Mice* ; Public Health Service, National Institute of Health: Research Triangle Park, NC, 2007.

(10) Chan, P.; Mahler, J.; Travlos, G.; Nyska, A.; Wenk, M. Induction of thyroid lesions in 14-week toxicity studies of 2- and 4-methylimidazole in Fischer 344/N rats and B6C3F1 mice. *Arch. Toxicol.* **2006**, *80*, 169–180.

(11) Chan, P.; Hill, G.; Kissling, G.; Nyska, A. Toxicity and carcinogenicity studies of 4-methylimidazole in F344/N rats and B6C3F1 mice. *Arch. Toxicol.* **2008**, *82*, 55–55.

(12) Chan, P.; Sills, R.; Kissling, G.; Nyska, A.; Richter, W. Induction of thyroid and liver tumors by chronic exposure to 2-methylimidazole in F344/N rats and B6C3F1 mice. *Arch. Toxicol.* **2008**, *82*, 399–412.

(13) Grosse, Y.; Baan, R.; Secretan-Lauby, B.; El Ghissassi, F.; Bouvard, V.; Benbrahim-Tallaa, L.; Guha, N.; Islami, F.; Galichet, L.; Straif, K. Carcinogenicity of chemicals in industrial and consumer products, food contaminants and flavourings, and water chlorination byproducts. *Lancet Oncol.* **2011**, *12*, 328–329.

(14) Office of Environmental Health Hazard Assessment. Chemicals Known to the State to Cause Cancer or Reproductive Toxicity, http://oehha.ca.gov/prop65/prop65_list/files/P65single010711.pdf (accessed Dec 28, 2011).

(15) Office of Environmental Health Hazard Assessment. No Significant Risk Level (NSRL) for the Proposition 65 Carcinogen 4-Methylimidazole, http://oehha.ca.gov/prop65/law/pdf_zip/1007114MEI.pdf (accessed Dec 28, 2011).

(16) European Food Safety Authority. Scientific Opinion of the ANS Panel: Re-evaluation of caramel colours (E 150 a,b,c,d) as food additives. *EFSA J.* **2011**, *9*, 2004–2106

(17) Fuchs, G.; Sundell, S. Quantitative determination of 4-methylimidazole as 1-acetyl derivative in caramel color by gas−liquid chromatography. *J. Agric. Food Chem.* **1975**, *23*, 120−122.
(18) Wilks, R. A. Jr.; Shingler, A. J.; Thurman, L. S.; Warner, J. S. The isolation of 4-methylimidazole from caramel color and its determination by thin-layer and gas−liquid chromatography. *J. Chromatogr., A* **1973**, *87*, 411−418.
(19) Wilks, R. A.; Johnson, M. W.; Shingler, A. J. An improved method for the determination of 4-methylimidazole in caramel color. *J. Agric. Food Chem.* **1977**, *25*, 605−608.
(20) Teixidó, E.; Santos, F. J.; Puignou, L.; Galceran, M. T. Analysis of 5-hydroxymethylfurfural in foods by gas chromatography-mass spectrometry. *J. Chromatogr., A* **2006**, *1135*, 85−90.
(21) Moon, J.-K.; Shibamoto, T. Formation of carcinogenic 4(5)-methylimidazole in Maillard reaction systems. *J. Agric. Food Chem.* **2010**, *59*, 615−618.
(22) Moretton, C. D.; Creétier, G. R.; Nigay, H.; Rocca, J.-L. Quantification of 4-methylimidazole in class III and IV caramel colors: validation of a new method based on heart-cutting two-dimensional liquid chromatography (LC-LC). *J. Agric. Food Chem.* **2011**, *59*, 3544−3550.
(23) Klejdus, B.; Moravcová, J.; Lojková, L.; Vacek, J.; Kubáň, V. Solid phase extraction of 4-methylimidazole (4-MI) and 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) from foods and beverages with subsequent liquid chromatographic-electrospray mass spectrometric quantification. *J. Sep. Sci.* **2006**, *29*, 378−384.
(24) Klejdus, B.; Moravcová, J.; Kubán, V. Reversed-phase high-performance liquid chromatographic/mass spectrometric method for separation of 4-methylimidazole and 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole at pg levels. *Anal. Chim. Acta* **2003**, *477*, 49−58.
(25) Rada-Mendoza, M.; Olano, A.; Villamiel, M. Determination of hydroxymethylfurfural in commercial jams and in fruit-based infant foods. *Food Chem.* **2002**, *79*, 513−516.
(26) Teixidó, E.; Moyano, E.; Santos, F. J.; Galceran, M. T. Liquid chromatography multi-stage mass spectrometry for the analysis of 5-hydroxymethylfurfural in foods. *J. Chromatogr., A* **2008**, *1185*, 102−108.