



Analytical Methods

Separation and determination of 4-methylimidazole, 2-methylimidazole and 5-hydroxymethylfurfural in beverages by amino trap column coupled with pulsed amperometric detection



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ABSTRACT

A method for simultaneous determination of 4-methylimidazole (4-Mel), 2-methylimidazole (2-Mel) and 5-hydroxymethylfurfural (HMF) in beverages was developed using solid-phase extraction (SPE) and amino trap column coupled with pulsed amperometric detection (AMTC-PAD). A single amino trap column (P/N: 046122) was first applied to separate the targeted analytes in samples after SPE pretreatment. This method demonstrated low limit of quantification (0.030 mg/L for methylimidazoles and 0.300 mg/L for HMF) and excellent linearity with correlation of determination ($R^2 = 0.999$ for 2-Mel, 0.997 for 4-Mel and 0.998 for HMF). Nearly no 2-Mel was found in all soft drinks. However, 4-Mel could be detected in cola drinks and soft drinks containing caramel colour (ranging from 0.13 to 0.34 mg/L), whereas HMF were only found in cola drinks (ranging from 1.07 to 4.47 mg/L). Thus, AMTC-PAD technique would be a valid and inexpensive alternative to analysis of 4-Mel, 2-Mel and HMF.

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1. Introduction

HMF, 4-Mel and 2-Mel were undesired byproducts produced during the manufacture of caramel colour (Capuano & Fogliano, 2011; Guan, Shi, Yu, & Xu, 2011; Guan, Wang, Yu, Yu, & Zhao, 2012; Hengel & Shibamoto, 2013; Moon & Shibamoto, 2010). HMF as a potential carcinogen was originated from the degradation of carbohydrate by the caramel reaction or Maillard reaction (Fig. 1) (Capuano & Fogliano, 2011; Delgado-Andrade, Seiquer, Haro, Castellano, & Navarro, 2010; Guan et al., 2011; Islam, Khalil, Islam, & Gan, 2014; Martins & Van Boekel, 2005). Methylimidazoles, formed from the reaction between dicarbonyl compounds (methylglyoxal or glyoxal) and ammonia in Maillard reaction (Fig. 1) (Jang, Jiang, Hengel, & Shibamoto, 2013; Lee, Jang, & Shibamoto, 2013), were confirmed as harmful compounds at a high dose exposure. Hengel and Shibamoto (2013) reported that the addition of caramel colour into beverages or soy sauces led to increase HMF, 4-Mel and 2-Mel concentration levels. Consequently, a great deal of attention was paid to the toxic, mutagenic,

and carcinogenic effects of these compounds in foods (Sengar & Sharma, 2012). Thus, a quantitative and routine analysis of HMF, 4-Mel and 2-Mel would be required for risk assessments and reliable exposure.

Traditionally, the separation and analysis of these polar compounds was accomplished by gas-liquid chromatography (GC) or reversed-phase liquid chromatography (RP-HPLC) method. For example, the acetyl derivatization of 4-Mel and 2-Mel were detected by a GC method (Casal, Fernandes, Oliveira, & Ferreira, 2002; Fuchs & Sundell, 1975). The determination of 4-Mel and 2-Mel in real foods were also performed by RP-HPLC method based on ion-pairing agent (Jang et al., 2013; Thomsen & Willumsen, 1981; Yamaguchi & Masuda, 2011). In addition, the determination of HMF was mainly carried out by RP-HPLC method (Delgado-Andrade et al., 2010). Wang and Schnute (2012) developed an improved method by an ultrahigh-performance liquid chromatography (UHPLC) tandem mass spectrometric (MS/MS) for the simultaneous quantification of 2-acetyl-4-tetrahydroxybutylimidazole (THI), 2-Mel, 4-Mel, and HMF in beverage samples, but the expensive instruments were not suitable for a small laboratory. Although these above-mentioned methods were applicable, stable and acute, a suitable modification of mobile phase or pre-column derivatization was required for the separation of polar compounds on GC and RP-HPLC column. Thus, a rapid and simple method for the routine

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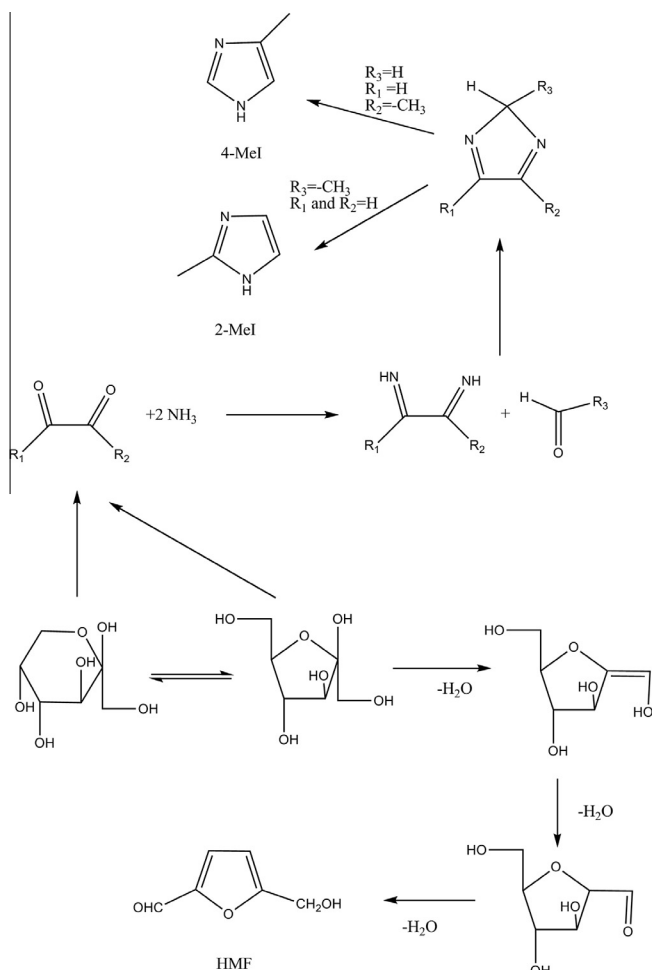


Fig. 1. Proposed mechanisms and the structures of 2-methylimidazole, 4-methylimidazole and 5-hydroxymethylfurfural formation.

analysis of 4-Mel and HMF was required. Methodologically, ion exchange chromatography, as a novel analytical technique first proposed by Small, Stevens, and Bauman (1975), was an ideal tool for the separation of ionic compounds or polar compounds without modification of mobile phase or pre-column derivatization. In this experiment, the target analytes as polar compounds (pKa values of the imidazole ring and HMF were 7.70 and 12.82, respectively.) were transformed into anionic forms in alkaline solution (Liu & Tan, 2013; Petrucci, Pereira, & Cardoso, 2013). Thus, the separation of 4-Mel, 2-Mel and HMF by anionic exchange chromatography method should be an optional strategy. However, to the best of our knowledge, the simultaneous separation of 4-Mel, 2-Mel and HMF as ionic forms was nearly no reported in literatures.

This study aimed to develop an applicable method for the simultaneous quantitation of HMF, 4-Mel and 2-Mel after solid-phase extraction (SPE) in real food system. The method was accomplished by a single amino trap column coupled with pulsed integrated amperometric electrochemical detector (AMTC-PAD). The results were discussed on selectivity, linearity, accuracy and precision of the method.

2. Materials and methods

2.1. Chemical and reagents

4-Mel (99%), 2-Mel (99%), 1, 2-dimethylimidazole (1,2-Mel, 99%), sodium hydroxide (50%) and HMF (98%) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Methanol,

ammonia and hydrochloric acid (HCl, 35–37%) were of analytic grade and purchased from Merck (Damstadt, Germany). The mixed standard stock solution of HMF (100 mg/L), 4-Mel (10 mg/L) and 2-Mel (10 mg/L) was prepared by dissolving the analytes into deionized water.

2.2. Samples preparation

The samples of cola and beverages were bought from local shops at Guang Zhou city. Soft drinks (a) with caramel colour and soft drink (b) and (c) without caramel colour had low levels of sugar. All the cola drinks contained caramel colour and only cola drink (e) did not contain sugar. Samples were degassed by a magnetic stirrer at room temperature for 30 min. A portion of drink (3 mL) was sampled before SPE. Class III caramel food colour (0.5 g/mL) was prepared and used as control to determine HMF, 4-Mel and 2-Mel.

2.3. Pretreatment of samples using SPE

Anslys SPEC SCX Disc 15 mg/3 mL cartridge (Varian, Walnut Creek, CA) was conditioned with 1 mL of methanol and 1 mL of water. The 3 mL samples (spiked 0.3 mg/L of 1,2-Mel as internal standard) acidified with 20 µL of HCl (0.1 mol/L) were loaded and passed through the cartridge. The retained imidazoles and HMF were eluted out with 6 mL of a methanol/ammonia (5%, v/v). The collected extract was evaporated to dryness at 39 °C and then the residues were dissolved with 3 mL deionized water for AMTC-PAD analysis.

2.4. AMTC-PAD conditions

AMTC-PAD was used for analysis of HMF, 4-Mel and 2-Mel. The samples were filtered through a Millex-HN nylon clarification kit of 0.45 µm pore size (Millipore, Bedford, MA), and analysed on a DX 5000 Dionex system (Dionex Corp., Sunnyvale, CA), a gradient pump (model EG40) with on-line degassing, and a pulsed integrated amperometric electrochemical detector (PAD, ED40, Dionex Corp., Sunnyvale, CA). Separation was accomplished on an amino trap column (4 × 50 mm, P/N: 046122) using the isocratic elution (100 mmol/L NaOH) for 60 min. All tests used a constant flow rate of 0.25 mL/min. The injection volume was 25 µL. The pH reference electrode (P/N: 061879) and gold working electrode (P/N: 061875) were used in AMTC-PAD analysis. The waveforms were applied under the following settings: $E_1 = 0.13$ V ($t_1 = 0.04$ s), $E_2 = 0.33$ V ($t_2 = 0.16$ s), $E_3 = 0.55$ V ($t_3 = 0.24$ s), $E_4 = 0.33$ V ($t_4 = 0.09$ s), $E_5 = -1.67$ V ($t_5 = 0.01$ s), $E_6 = 0.93$ V ($t_6 = 0.01$ s), $E_7 = 0.13$ V ($t_7 = 0.01$ s). Integration occurred from 0.20 to 0.44 s during E_3 application.

The calibration curves were listed in Table 1. The regression equation for HMF was $y_1 = 2.493x_1 + 3.891$; $R^2 = 0.998$, where x_1 = HMF concentration, mg/L; y_1 = peak area of HMF. The regression equation for 4-Mel was $y_2 = 16.480x_2 + 0.479$; $R^2 = 0.997$, where x_2 = 4-Mel concentration, mg/L; y_2 = peak area of 4-Mel. The regression equation for 2-Mel was $y_3 = 9.354x_3 + 0.470$; $R^2 = 0.999$, where x_3 = 2-Mel concentration, mg/L; y_3 = peak area of 2-Mel.

According to Sistla, Tata, Kashyap, Chandrasekar, and Diwan (2005), limit of detection (LOD) and limit of quantification (LOQ) was determined as three times and ten times observed signal-to-noise (S/N), respectively.

2.5. Recovery tests

The recoveries were calculated by comparing the peak areas of the spiked and non-spiked samples with the peak areas of standard

Table 1Linear calibration ranges and regression equations of analytes as measured by AMTC-PAD.^b

Analytes ^c	Calibration range (mg/L)	Regression equation, y^a	Coefficient regression, R^2	RSD (%), $n = 6$
HMF	1.000–100.000	$y = 2.493x + 3.891$	0.998	2.10
4-Mel	0.100–10.000	$y = 16.480x + 0.479$	0.997	1.27
2-Mel	0.100–10.000	$y = 9.354x + 0.470$	0.999	1.73

^a x , concentration of analytes (mg/L); y , peak area of analytes (nC*min).^b AMTC-PAD, amino trap column with pulsed integrated amperometric electrochemical detector.^c HMF, 4-Mel and 2-Mel represented for 5-hydroxymethylfurfural, 4-methylimidazole and 2-methylimidazole, respectively.

solutions at the same concentration, the value of recovery was calculated as followed: $\text{recovery (\%)} = \frac{A_S - A_{NS}}{A_{SS}} \times 100$. Where A_S = peak area of spiked sample, A_{NS} = peak area of non-spiked samples, and A_{SS} = peak area of standard solution.

2.6. Precision and accuracy

Precision was calculated as the relative standard deviation (RSD) and the accuracy (%) was calculated by observed amount/specified amount $\times 100\%$.

2.7. Statistical analysis

Means and standard deviations of the data were calculated for each treatment by the SPSS software package (SPSS 10.0 for windows).

3. Result and discussion

3.1. AMTC-PAD system

In this experiment, 4-Mel, 2-Mel and HMF were deprotonated into their relative anionic forms in basic solution and thereby could be retained on an anion exchange analytical column. However, when the separation of 4-Mel, 2-Mel and HMF was performed on a conventional anion exchange column (such as AS23, CarboPac PA1, AminoPac PA10 or CarboPac PA20 columns), a poor peak resolution and a short retention time ($rt.$) were obtained. Moreover, the analysis of the target analytes was interfaced by the residual matrix of food after SPE (data not shown). Thus, a conventional anion exchange column was not applicable for the simultaneous analysis of target analytes. Fortunately, an improved method (high peak resolution and appropriate retention time) was developed by an amino trap column for the separation of the anionic 2-Mel, 4-Mel and HMF. Dionex document (Document No. 031197-5) introduced that the amino trap column was packed with a 10 μm , high capacity anion exchange resin which had high selectivity for hydrophobic amino acids relative to monosaccharides, using hydroxide eluents. Interesting, the amino trap similarly exerted excellent selectivity for 4-Mel, 2-Mel and HMF on the basis of experiment data. Therefore, the desired chromatograph system was accomplished by a PAD and a single amino trap column, not required other anion exchange analytical column. In addition, a pressurizer, replaced by a 50 cm PEEK tubing (internal diameter = 0.01 mm) before the six-port valve, was required to maintain the pressure of system at 6 Mpa (flow rate of 0.25 mL/min) and to minimise the noise of the system. In application, the commercial amino trap column was cheaper than the C18 column or GC column for 4-Mel analysis. In addition, no other modifier reagent (like ion-pair reagent) should been added into the mobile phase during the separation of target analytes. Obviously, the developed AMTC-PAD system for analysis of 4-Mel was simple and inexpen-

sive with respect to the HPLC-MS or GC-MS systems for 4-Mel analysis.

3.2. Method development

Fig. 2 showed AMTC-PAD chromatograms of HMF, 4-Mel and 2-Mel in different samples separated by an amino trap column. Based on our previous research, the low concentration of NaOH was not suitable for the separation of 4-Mel on an amino trap column. Moreover, the peak of 4-Mel was irregular under the low concentration of NaOH. The reason was that 4-Mel was not well deprotonated in the low concentration of NaOH and thereby the partly ionic 4-Mel affected the peak shape and retention of 4-Mel on the amino trap column. Finally, 100 mM NaOH was assigned as the appropriate eluent to separate the target analytes. Besides, 1, 2-Mel in a constant amount (0.300 mg/L), as the internal standard, was spiked into samples to correct for the loss of analyte during sample preparation or sample inlet. As shown in Fig. 2, peak 1, peak 2, peak 3 and peak 4 was presented for the peak of 2-Mel ($rt.$ = 9.8 min), 4-Mel ($rt.$ = 18.9 min), HMF ($rt.$ = 33.5 min), 1,2-Mel ($rt.$ = 41.3 min), respectively. 1,2-Mel, as a suitable internal standard, had a strong retention on amino trap column and little interface with the compounds in samples. The impurities of samples had a short retention on the amino trap column and were totally eluted out before 8.0 min. In addition, the impurities co-eluted with target analytes could also be identified by the spiked standard into samples. For example, chromatograms F and G in Fig. 2 obtained from soft drinks (a) and (c) had a peak at $rt.$ = 10.2 min, which was difficult to distinguish with the peak of 2-Mel. However, the co-elute peak could be identified in chromatograms obtained from the spiked samples. As shown in chromatograms C and D in Fig. 2, after 2-Mel standard solution was spiked into soft drinks (a) and (c), a shoulder peak was shown on peak at 10.2 min. The result suggested that the peak at 10.2 min in chromatograms F and G could be identified as the peak of impurity compound instead of 2-Mel.

Besides, the target analytes in the beverages after SPE pretreatment were separated well from the matrix of beverages (chromatography E, F and G in Fig. 2). Meanwhile, all peaks of the target analytes were symmetrical and had no tail. Meanwhile, the resolution (r) among the target analytes was larger than 3.0. Especially, the amino trap column showed a well behaviour during separation of imidazoles. A relatively high resolution of the imidazoles could be obtained in the AMTC-PAD method with respect to the prior method for 4-Mel analysis. For example, when 4-Mel in caramel colour was analysed using capillary isotachopheresis (CE) method, the highest resolution of the imidazoles ($r = 2$) was lower than the value obtained from the AMTC-PAD method (Petruci et al., 2013). Similarly, the resolution of the imidazoles obtained from RP-HPLC method was undesirable. As a result, the LOD of 4-Mel in HPLC-DAD method was insufficient to detect the levels occurring in beverage samples, even when SPE was applied for sample cleanup and

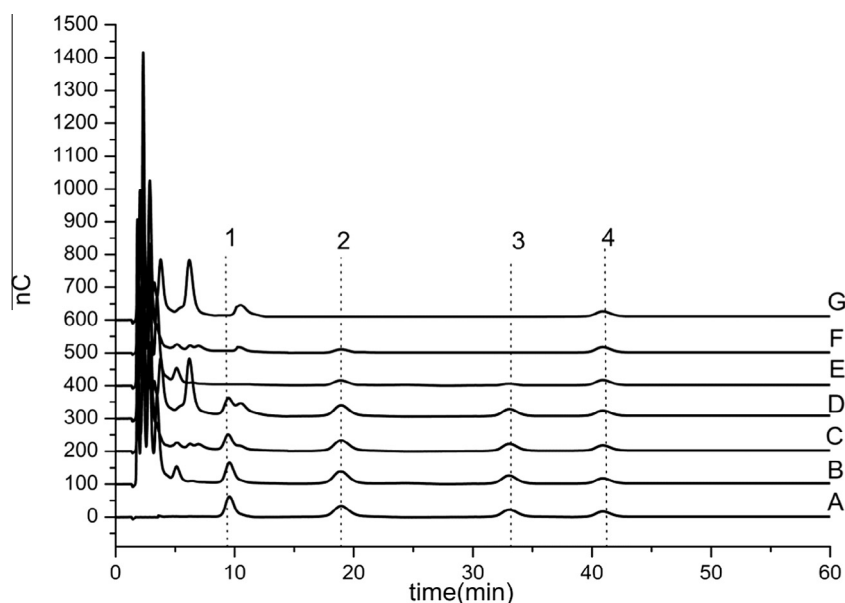


Fig. 2. AMTC-PAD chromatograms of blank and beverages samples spiked standard analytes (0.300 mg/L 2-methylimidazole, 0.300 mg/L 4-methylimidazole, 3.000 mg/L 5-hydroxymethylfurfural and 0.300 mg/L 1,2-dimethylimidazole) after solid-phase extraction. (A) Standard analytes after solid-phase extraction; (B) extracts after solid-phase extraction in cola a spiked standard analytes; (C) extracts after solid-phase extraction in soft drink a spiked standard analytes; (D) extracts after solid-phase extraction in soft drink c spiked standard analytes. (E) extracts after solid-phase extraction in cola a. (F) Extracts after solid-phase extraction in soft drink a. (G) Extracts after solid-phase extraction in soft drink c. Peak 1, 2, 3 and 4 represented for the peaks of 2-methylimidazole, 4-methylimidazole, 5-hydroxymethylfurfural and 1,2-dimethylimidazole (an internal standard), respectively.

pre-concentration. For this reason, the analysis of 4-Mel on RP-HPLC system had to switch to using the expensive HPLC–MS/MS method to overcome the matrix effect (Schlee et al., 2013).

3.3. Method performance

Linearity and calibration, correlation of determination (R^2), detection limit, precision, accuracy and recovery were mainly evaluated as quality parameters of AMTC-PAD method.

Calibration curves were generated from calibration standards (HMF ranging from 1.000 to 100 mg/L and methylimidazoles ranging from 0.100 to 10 mg/L). As shown in Table 1, calibration equations of both methylimidazoles and HMF had excellent linearity with the correlation of determination for HMF ($R^2 > 0.998$), 2-Mel ($R^2 > 0.999$) and 4-Mel ($R^2 > 0.997$). In this study, LOQ was determined as ten times observed S/N. LOQ observed for all analytes was shown in Table 2. LOQ of HMF, 4-Mel and 2-Mel was 0.300, 0.045 and 0.060 mg/L, respectively. Similarly, LOD of HMF, 4-Mel and 2-Mel was 0.100, 0.015 and 0.020 mg/L, respectively. The result suggested that HMF had a relatively low LOD with respect to methylimidazoles since HMF with only one hydroxyl group had weak signal in PAD.

Compared with the previously routine methods for 4-Mel analysis, ultraviolet detector (UV) or differential refraction detector (RID) coupled with RP-HPLC or CE was mainly applied for 4-Mel

determination. Due to the matrix effect, LODs of 4-Mel in these methods were unsatisfied (about 0.1 mg/L for CE-UV and 1 mg/L for HPLC-UV) (Ciolino, 1998; Petrucci et al., 2013; Schlee et al., 2013). Thus the prior methods were not suitable for 4-Mel analysis at trace concentration. On the contrary, because of the high resolution and little matrix effect in AMTC-PAD method, the economical PAD could be directly applied for quantification of 2-Mel, 4-Mel and HMF.

In addition, the precision and accuracy, evaluated by six replicate assays of standard at 1.000 mg/L, were addressed by %RSD and %accuracy, respectively. As shown in Table 2, excellent precision was observed for all analytes with %RSD < 5%. Accuracy ranging from 95% (2-Mel) to 99% (4-Mel) indicated accurate measurements could be achieved using the method in this experiment.

Recovery was evaluated by spiking target analytes at three concentrations ($n = 3$) in cola drinks and soft drinks. The results were shown in Table 3. Observed recoveries ranged from 95.9% (2-Mel, 0.300 mg/L spiked) to 106.2% (HMF, 1.500 mg/L spiked).

3.4. Analysis of HMF, 4-Mel and 2-Mel in beverages

The analysis was performed in cola and soft drinks using the proposed method. The results were listed in Table 4. The identification of HMF, 4-Mel and 2-Mel was confirmed by spiking standards

Table 2
Precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).^c

Analyte	1.000 mg/L ($n = 6$)		LOD (mg/L) ^a	LOQ (mg/L) ^b
	Accuracy (%)	RSD (%)		
5-Hydroxymethylfurfural	98	4.68	0.100	0.300
4-Methylimidazole	99	3.73	0.015	0.045
2-Methylimidazole	95	4.56	0.020	0.060

^a S/N = 3, per 20 μ L injection volume.

^b S/N = 10, per 20 μ L injection volume.

^c Five data points, with three replicate injections at each concentration level. RSD, relative standard deviation.

Table 3Recovery of target analytes.^a

Spiked concentration (mg/L)	4-Methylimidazole			2-Methylimidazole			5-Hydroxymethylfurfural		
	0.150	0.300	0.450	0.150	0.300	0.450	1.500	3.000	4.500
Cola drink	103.4(4.15)	98.3(5.13)	95.1(4.41)	104.1(4.12)	95.9(3.23)	97.2(5.63)	106.2 (5.13)	99.3 (6.53)	101.4 (5.43)
Soft drink	99.6(5.33)	102.2(6.02)	103.1(6.96)	97.9(3.45)	101.8(2.69)	104.4(4.87)	101.1 (3.32)	105.3(4.22)	99.9(4.38)

^a Results shown were %recovery with %RSD in parentheses.**Table 4**

Concentrations of imidazoles and 5-hydroxymethylfurfural in soft drinks.

Samples	4-Methylimidazole (mg/L)	2-Methylimidazole (mg/L)	5-Hydroxymethylfurfural (mg/L)
Cola a	0.23 ± 0.46	<LOD	1.10 ± 0.73
Cola b	0.16 ± 0.11	<LOD	3.40 ± 0.52
Cola c	0.32 ± 0.44	<LOD	4.47 ± 0.76
Cola d	0.34 ± 0.56	<LOD	1.07 ± 0.30
Cola e	0.13 ± 0.25	<LOD	1.82 ± 0.27
Soft drink a (contain caramel colour)	0.15 ± 0.28	<LOD	<LOD
Soft drink b (no caramel colour)	<LOD	<LOD	<LOD
Soft drink c (no caramel colour)	<LOD	<LOD	<LOD

into the extracts. In five cola drinks, nearly no 2-Mel was found in all samples and low concentration level of 4-Mel and HMF was present in these drinks (highest level, 0.34 ± 0.56 mg/L for 4-Mel and 4.47 ± 0.76 mg/L for HMF). However, HMF and methylimidazoles were nearly not present in the soft drinks except the soft drink containing caramel colour (0.15 mg/L 4-Mel). The result suggested that the presence of harmful compounds in beverages may be resulted in by the addition of caramel colour. Lastly, the result of 4-Mel was in agree with the prior studies for analysis of 4-Mel in foods (Hengel & Shibamoto, 2013; Wang & Schnute, 2012). Thus, the newly developed method allowed the stable and simultaneous analysis of HMF, 2-Mel and 4-Mel in beverages after SPE separation.

4. Conclusions

The simultaneous analysis of 4-Mel, 2-Mel and HMF after SPE pretreatment was firstly accomplished by AMTC with PAD. Meanwhile, the proposed method had the advantages of excellent peak resolution, good linearity and appropriate accuracy. It was a suitable method for routine analysis of 4-Mel, 2-Mel and HMF in beverages.

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