Revised method for analyzing 2-acetyl-4-tetrahydroxybutylimidazole in caramel III

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Abstract

Caramel III, a food-coloring additive, is tested in Japan for the presence of the impurity, 2-acetyl-4-tetrahydroxybutylimidazole (THI), using an official HPLC method. In this HPLC method, THI is derivatized with 2,4-dinitrophenylhydrazine and then separated using octyl column. Improvement of the analytical conditions was attempted because contaminants can often compromise this test. Isolation of the analyte was improved when 0.1 mol/L phosphoric acid/methanol mixed solution (70:30) was used as the mobile phase. The revised method gave higher analyte concentrations compared to the standard method. The quantitative values obtained by LC/MS were equivalent to those obtained using the revised method, demonstrating the superiority of the revised method to the standard method.

Keywords: caramel, 2-acetyl-4-tetrahydroxybutylimidazole, 2,4-dinitrophenylhydrazine, HPLC, octyl column

I Introduction

Caramel III, a food-coloring additive, is tested in Japan for the presence of the impurity, 2-acetyl-4-tetrahydroxybutylimidazole (THI), using an official HPLC method. This method requires the derivatization of THI with 2,4-dinitrophenylhydrazine (DNPH, Fig. 1)3-5. THI is reported to have immunotoxicity, such as a lymphopenic effect in rats.4,5) The standard method is based on the method established by Kröplien et al.6) Similar methods are defined under standards of JECFA, EU, and FCC.7,8)

The method comprises purifying THI from caramel III on a column containing two kinds of cation exchange resins, reacting THI with DNPH to derivatize it to hydrazone (THI-DNPH, Fig. 1), and then using an octyl column to isolate and quantify THI-DNPH by HPLC using 0.1 mol/L phosphoric acid/methanol (50:50, v/v) as the mobile phase. However, using the official method, the separation of THI from contaminants is often poor, and the reliability of the quantitative values has been proven problematic. Also, although the JECFA standard recommends an HPLC octyl column with 10 µm particle size,
the HPLC octyl columns currently commercially available generally have 5 μm particle size.

In order to establish a more reliable method for analyzing THI using an HPLC octyl column that is widely available, we investigated the effects of the mobile phase and the type of column on the isolation of THI-DNPH from contaminants, and on the reliability of the obtained quantitative values.

II Materials and Methods

1. Samples, reagents, and instrumentation
Caramel III products 1 to 6 and THI were procured from the Japan Caramel Industrial Association. Reagent-grade phosphoric acid, DNP, methanol and ethanol were used. The HPLC solvent used HPLC-grade components. A combination column meeting the official standards was used, with Amberlite CG-50 in the upper part of the column and Dowex 50W-X8 in the lower part of the column. Both resins were purchased from Wako Pure Chemical Industries.

2. Preparation of the test solution and THI-DNPH
THI-DNPH was synthesized from THI according to the official method. The THI-DNPH was dissolved in methanol to provide 0, 10, 20, or 40 μg/mL standard solutions.

3. HPLC analysis conditions
The HPLC system used was composed of an Alliance Separations Module 2695 and a Photodiode Array Detector 2996 (Waters). Three kinds of octyl column were used: Column A was a LiChrosorb RP-8, 4.6 mm i.d. × 250 mm, 10 μm particle size (GL Science); column B was a TSKgel Octyl-80Ts, 4.6 mm i.d. × 250 mm, 5 μm particle size (Tosoh), and column C was a Wakosil-II 5C8 H8, 4.6 mm i.d. × 250 mm, 5 μm particle size (Wako Pure Chemical Industries). The column was used under room temperature or at 30°C. The mobile phase was a 0.1 mol/L phosphoric acid/methanol mixed solution with a solvent ratio of 50:50 (v/v), 60:40 (v/v), 62:38 (v/v), 64:36 (v/v), 66:34 (v/v), 68:32 (v/v) or 70:30 (v/v). The flow rate was adjusted in column A to 0.7 mL/min, so that the THI-DNPH retention time would be 6.3 ± 0.1 min; in columns B and C, it was set to 0.8 mL/min. A wavelength of 385 nm was used for quantification.

4. LC/MS analysis conditions
LC/MS was performed using an ACQUITY UPLC/SQD system (Waters). Three kinds of ODS column were used: Column D was an L-column2 ODS, 2.1 mm i.d. × 150 mm, 3 μm particle size (Chemicals Evaluation and Research Institute), column E was an Inertsil ODS-4, 2.1 mm i.d. × 150 mm, 3 μm particle size (GL Science), and column F was a TSKgel ODS-100V, 2.0 mm i.d. × 150 mm, 3 μm particle size (Tosoh). Operating conditions were: column temperature 30°C, mobile phase: 0.1% formic acid/methanol mixed solution (70:30, v/v); flow rate: 0.2 mL/min; ionization: ESI positive; capillary voltage: 3.0 kV; cone voltage: 30 V; source temperature: 110°C; desolvation temperature: 350°C; desolvation gas flow rate: 800 L/hr; cone gas flow rate: 30 L/hr; detection: SIR mode, m/z 411.

III Results and Discussion

1. Effect of mobile phase composition ratio
Column A was an octyl column containing 10 μm diameter particles and column B and column C were octyl columns containing 5 μm particles. Recently, columns containing 5 μm and smaller particles have become generally available; therefore, the mobile phase composition ratio was examined using column B. The column was used under room temperature. The test solution was prepared from sample 5, shown by preliminary studies to have the highest THI content.

Fig. 2 shows a chromatogram of the test solution. Analysis using the 0.1 mol/L phosphoric acid/methanol mixed solution (50:50, v/v) as the mobile phase, used in the standard Japanese testing method, resulted in the elution of the THI-DNPH peak after the baseline was elevated and the incomplete separation of the THI-DNPH peak with a contaminant peak. This raised concerns regarding the reliability of the quantitative values. The reproducibility of the HPLC profile indicated that the contaminants were derived from the caramel. As the methanol content ratio of the mobile phase was decreased, the baseline became flatter and the THI-DNPH peak was separated from a contaminant peak, peak B, which eluted immediately posterior to the THI-DNPH. Another contaminant, peak A, was detected immediately prior to the THI-DNPH peak at a phosphoric acid/methanol ratio of 62:38 (v/v). A solvent ratio of 64:36 (v/v) provided better separation of the THI-DNPH peak and peak A, and at solvent ratios of 66:34 (v/v) and 68:32 (v/v), baseline separation was achieved (Fig. 2). The PDA spectrum of peak A showed a maximum absorption wavelength of 359 nm, whereas that of THI-DNPH was 385 nm; thus, peak A is unlikely to be a DNPH-derivatized compound (Fig. 3).

The THI-DNPH concentration in the test solution was determined from the peak areas for the reference and test solutions for each of the mobile phases and the THI content in the sample was calculated (Table 1). At a solvent ratio of 60:40 (v/v), which resulted in co-elution of the THI-DNPH peak and contaminant peak A, the quantitative value was calculated to be 46 μg/g. In contrast, at solvent ratios of 62:38 (v/v), 64:36 (v/v), 66:34 (v/v), 68:32 (v/v), 70:30 (v/v), which allowed separation of the THI-DNPH and contaminant A peaks, the
THI concentration was calculated to be consistently between 30 to 34 μg/g. These results suggest that a solvent ratio of 70:30 (v/v) would allow complete separation of the THI-DNPH peak and the contaminant peak A, and thus would allow more accurate quantitation of THI.

2. Comparison of quantitative values from the official method, the revised method, and the LC/MS method

Three test solutions were prepared from sample 5 according to the official method. The samples were passed through column A, containing 10 μm particles, with the mobile phase of the official method at a flow rate of 0.7 mL/min. The column temperature was set to 30°C. Under this condition, the retention time of THI-DNPH was 6.3 ± 0.1 min and met the criterion of the official method. The same three test solutions were passed through column B and column C, which have a particle size of 5 μm, with the revised mobile phases. Fig. 4 shows typical chromatograms provided by the official test method and the revised mobile phases with the two types of columns. With the official method and column A, the THI-DNPH peak was eluted on an elevated baseline, whereas with the revised method the baseline was flat. When the THI content in the samples was calculated, the method using the revised mobile phases provided higher quantitative values for all test solutions compared to the official method (Table 2). Average quantitative values and standard deviations (SDs) of three test solutions are shown in Table 3. The small standard deviation for all quantitative values suggests that the sample preparation methodology in the official test method is not problematic.

Next, LC/MS was used to confirm the validity of the obtained quantitative values. The mobile phase used in the official testing method, the revised method, and the method in the report using an ODS column9 contained high concentrations of phosphoric acid. This non-volatile acid is not suitable for LC/MS, and so was replaced with 0.1% formic acid. Quantification using the three types of ODS column was studied. The quantitative values for THI-DNPH in the

| Table 1. Retention time of THI-DNPH and amount of THI. |
|---------------------------------|-----------------|---------------|
| Solvent ratio*                  | Retention time of THI-DNPH (min) | Amount of THI (μg/g) |
| (v/v)                           |                               |                |
| 50 : 50                         | 5.30                          | 34.39          |
| 60 : 40                         | 7.41                          | 45.50          |
| 62 : 38                         | 8.15                          | 31.59          |
| 64 : 36                         | 9.03                          | 30.46          |
| 66 : 34                         | 10.06                         | 34.40          |
| 68 : 32                         | 11.39                         | 29.83          |
| 70 : 30                         | 12.96                         | 32.77          |

* 0.1 mol/L phosphoric acid : methanol.

Fig. 2. HPLC chromatograms of test solutions separated on column B. The column temperature was set to room temperature. The ratio of 0.1 mol/L phosphoric acid to methanol in the mobile phase is shown in the upper right of each chromatogram.

Fig. 3. Spectra of THI-DNPH and Peak A. The maximum absorption wavelength in each spectrum is indicated with an arrow.
Fig. 4. HPLC chromatograms of the test solution obtained using the official and revised methods. The baseline for the THI-DNPH peak is shown in each chromatogram. Upper, official method; middle and lower, revised method.

Table 2. Amount of THI determined by the official method and the revised method. Average and SD of three injections are shown.

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Method</th>
<th>Column</th>
<th>Amount of THI (µg/g, average ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Official</td>
<td>A</td>
<td>25.55 ± 1.44</td>
</tr>
<tr>
<td></td>
<td>Revised</td>
<td>B</td>
<td>28.33 ± 0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>28.08 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>Official</td>
<td>A</td>
<td>25.96 ± 2.33</td>
</tr>
<tr>
<td></td>
<td>Revised</td>
<td>B</td>
<td>29.88 ± 0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>27.73 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>Official</td>
<td>A</td>
<td>23.08 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>Revised</td>
<td>B</td>
<td>31.74 ± 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>26.80 ± 0.09</td>
</tr>
</tbody>
</table>

three test solutions were determined by UV at 385 nm and also with the mass spectrometer in ESI positive mode. UV detection resulted in elevated baseline and poor separation from contaminant peaks. However, in the mass detection, SIR measurement of the [M+H]⁺ ion peak at m/z 411 provided a single peak (Fig. 5). The THI-DNPH concentration in the test solutions was calculated by the absolute calibration method from the THI-DNPH peak area obtained by SIR measurements. The same concentration of THI in the samples was calculated from data obtained using the three types of ODS column (Table 3).

Comparison of the quantitative values obtained by LC/MS, the official method and the revised method showed that the values obtained using the official method are about 80% of those obtained using LC/MS. In contrast, the revised method provides values equivalent to those obtained using LC/MS (Table 3).

As shown above, the Japanese standard test method for THI in caramel III provides poor separation of the THI peak.

Fig. 5. LC/MS chromatograms of the test solution separated on column F. Upper, UV chromatogram at 385 nm; Lower, mass chromatogram (MC) at m/z 411.

Table 3. Amount of THI determined by the official method, the revised method and the LC/MS method. Three test solutions were analyzed independently.

<table>
<thead>
<tr>
<th>Method</th>
<th>Column</th>
<th>Amount of THI (µg/g, average ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Official</td>
<td>A</td>
<td>24.86 ± 1.56</td>
</tr>
<tr>
<td>Revised</td>
<td>B</td>
<td>29.99 ± 1.71</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>27.54 ± 0.66</td>
</tr>
<tr>
<td>LC/MS</td>
<td>D</td>
<td>30.24 ± 1.61</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>31.32 ± 1.57</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>31.88 ± 1.51</td>
</tr>
</tbody>
</table>
from a contaminant peak. In an effort to improve quantitative accuracy, the mobile phase was optimized. LC/MS was used to verify the reliability of the quantitative values from the optimized revised method. The results indicate that more accurate quantitative values can be calculated from data obtained using the revised method than is possible using with the official test method.

IV Conclusion

The Japanese standard test method for quantifying THI in caramel III requires the derivatization of THI to THI-DNPH, followed by separation by HPLC on an octyl column using a 0.1 mol/L phosphoric acid/methanol mixed solution as the mobile phase. Lowering the methanol content in the mobile phase to 30v/v% flattened the baseline and provided superior separation of the THI-DNPH peak from the contaminant peak. Quantitative values determined using this revised method were equal to those obtained using LC/MS, suggesting that the revised method will allow more accurate quantitation of THI.

V Acknowledgements

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VI References

ノート

カラメルⅢ中の2-アセチル-4-テトラヒドロキシンプチルイミダゾール分析法の改良
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キーワード: カラメル、2-アセチル-4-テトラヒドロキシンプチルイミダゾール、2,4-ジニトロフェニルヒドラジン、HPLC、オクチルカラム

概要
カラメルⅢに含まれる2-アセチル-4-テトラヒドロキシンプチルイミダゾールを、2,4-ジニトロフェニルヒドラジンと反応させた後にHPLCで分離して定量する純度試験において、第8版食品添加物公定書の方法では夾雑物との分離が不十分であるため、HPLCの溶媒比の変更を検討した。溶媒として0.1mol/Lリン酸／メタノール混液（70:30, v/v）を用いる時に良好な分離が得られた。本条件による定量値を8版記載条件と比較し、さらに0.1％酸／メタノール混液（70:30, v/v）を溶媒としたLC/MSによる定量値と比較した。改良法はLC/MS法と近い値を示した。