Japan Customs Analysis Methods

No. 112

Quantitative Analysis of Cocoa
(Issued in July 1999)
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1. Scope

This analysis method is applied to food preparations containing cocoa which require the determination of the cocoa contents (calculated on a totally defatted basis) as specified in Note 3 of Chapter 19 and the term of heading 19.01, of Customs Tariff law (Appendix Table - Customs Tariff Schedule).

2. Outline of Test Method

This method is used for determination of the cocoa contents in foods containing cocoa based on the measured theobromine and caffeine contents. The procedure is as follows.

(1) Extraction of alkaloids from foods containing cocoa.
(2) Determination of the theobromine and caffeine contents using a high performance liquid chromatograph.
(3) Calculation of cocoa content (on a totally defatted basis) on the basis of the theobromine and caffeine contents

3. Apparatus

High performance liquid chromatograph (HPLC), equipped with a detector to measure absorbance at 273 nm (e.g. UV detector). Measurement conditions are as follows:

(a) Separation column, Zorbax ODS, 4.6 mm I.D. x 250 mm, or equivalent column
(b) Column temperature, 25°C
(c) Mobile phase, water : acetonitrile (85 : 15) (Both HPLC grade)
(d) Flow rate, 1.0 ml/min
(e) Detection, UV 273 nm
(f) Injection volume, 20 µl

Note 1) The column temperature, mobile phase and flow rate, etc. may be changed depending on the column used. (For example, conditions for a similar column can be applied with the following settings: mobile phase, water: tetrahydrofran (100:1); column temperature, 45°C; flow rate, 1.5 ml/min; etc.)

4. Reagents

All chemicals must be JIS special reagent grade or equivalent, unless otherwise specified.

(a) Theobromine
(b) Caffeine
(c) β-Hydroxyethyltheophylline (1,3-Dimethyl-7-β-[2-hydroxyethyl]xanthine)
(d) Petroleum ether
(e) Deproteinizing agent

A: Dissolve 2 g of zinc sulfate heptahydrate (ZnSO₄·7H₂O) in water and bring to 100 ml volume with water.
B: Dissolve 1.8 g of barium hydroxide octahydrate [Ba(OH)₂·8H₂O] in water and bring to 100 ml volume with water.

5. Preparation of standard solutions for calibration curve

(1) Theobromine standard stock solution

Accurately weigh approximately 100 mg of theobromine to a beaker. Transfer it with water into
a 1,000-ml brown volumetric flask and dissolve. Dilute to volume with water. Use this solution as the theobromine standard stock solution.

(2) Caffeine standard stock solution

Accurately weigh approximately 50 mg of caffeine to a beaker. Transfer it with water to a 1,000-ml brown volumetric flask and dissolve. Dilute to volume with water. Use this solution as caffeine standard stock solution.

(3) Internal standard solution

Accurately weigh approximately 100 mg of β-Hydroxyethyltheophylline to a beaker. Transfer it with water to a 1,000-ml brown volumetric flask and dissolve. Dilute to volume with water. Use this solution as internal standard solution.

(4) Standard solutions for calibration curve

By means of whole pipets, add 5, 10, 15, 20, 30 and 50 ml of the theobromine standard stock solution (prepared in (1)) to separate 200-ml brown volumetric flasks. Then, add 1, 2, 4, 6, 8 and 10 ml of the caffeine standard stock solution (prepared in (2)) to the respective flasks. To each volumetric flask, add 20 ml of the internal standard solution (prepared in (3)) by a whole pipet. Dilute to volume with water. Use these as standard solutions for constructing calibration curves of theobromine and caffeine.

6. Preparation of Sample and Test solution

6.1. Preparation of Sample

Test samples should be prepared using appropriate methods such as the reduction method depending on their states. For solid test materials such as chocolates, chill them until hard, and grate or shave to fine granular condition. For paste or wet test materials, homogenize them using a mortar and pestle. In either case, a relatively large quantity of test materials must be sampled randomly and then homogenized by mixing and/or pulverizing.

6.2. Preparation of Test Solution

Accurately weigh the test sample prepared in 6.1. to a large centrifuge tube (50 ml or larger capacity). Add 30 ml of petroleum ether and stir thoroughly. Centrifuge the mixture at 5,000 rpm (approximately 2,800×g) for 10 min. Add 10 ml of water and centrifuge at 7,500 rpm (approximately 6,300×g) for 15 min. After centrifugation, discard the upper layer of petroleum ether using a pipette or other tools. (3)

Transfer the lower layer containing residues in the centrifuge tube to a 200-ml Erlenmeyer flask. Wash the residues with water sufficiently and transfer all the washings to the Erlenmeyer flask above. Then, add water to make a total of approximately 100 ml volume.

Heat the flask in a boiling water bath for 25 min, stirring occasionally.

After cooling, add precisely 20 ml of the internal standard solution using a whole pipette and mix. Add 10 ml each of deproteinizing agents A and B and mix. Leave to stand for a while, and add water to make a total of approximately 200 ml volume.

Heat the solution (approximately 200 ml volume) in the flask again in a boiling water bath for 10 minutes, stirring occasionally. Filter the contents through filter paper (e.g., JIS P 3801 Class 5, No. 5C) while still warm. Filter again through a membrane filter of a 0.45 μm pore-size. Inject the filtrate into HPLC.

Note 2) Since theobromine is poorly soluble in water, test solution should be prepared with a theobromine concentration of 0.5 mg/ml or lower. The approximate quantities of test materials to be sampled are shown in the table below. For other foodstuffs, determine the amount of test material to be sampled through backward calculation based on the cocoa content.

<table>
<thead>
<tr>
<th>Kinds of substances</th>
<th>Sampling Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa powder</td>
<td>0.1</td>
</tr>
<tr>
<td>Cacao mass</td>
<td>0.2</td>
</tr>
<tr>
<td>Chocolate</td>
<td>0.3</td>
</tr>
<tr>
<td>Milk chocolate</td>
<td>0.3</td>
</tr>
<tr>
<td>Chocolate sandwich</td>
<td>0.5</td>
</tr>
<tr>
<td>Chocolate milk</td>
<td>1.0</td>
</tr>
<tr>
<td>Chocolate ice cream</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Note 3) For test materials not containing fat or not dispersing in petroleum ether, these defatting steps can be omitted.
7. Procedure

7.1. Preparation of calibration curve

Inject 20 μl of each standard solution prepared in 5 (4) into HPLC. From the obtained chromatogram, measure the peak areas of theobromine, caffeine and internal standard substances.

Draw the calibration curves of theobromine and caffeine by plotting the weight ratio (Wx/Ws) of each substance (Wx) to the internal standard substance (Ws) against the peak area ratio (Ax/As) of each substance (Ax) to the internal standard substance (As). (See graphs 1 and 2 below.)

\[
\% \text{, theobromine (or \% caffeine) } = \frac{(Wx/Ws) \times Ms}{S \times 100}
\]

Where -

- Wx/Ws: Weight ratio of theobromine or caffeine to internal standard substance, obtained from the calibration curve
- Ms: mg of the internal standard substance per 20 ml of internal standard solution
- S: Sample weight (g)

7.2. Determination of theobromine and caffeine in test solution

Inject 20 μl of the test solution prepared in 6.2 into HPLC. Calculate the peak area ratios of theobromine and caffeine to the internal standard substance based on the peak areas of each substance from the obtained chromatogram, and convert them to weight ratios using the calibration curves constructed in 7.1.

Calculate the contents of theobromine and caffeine in the test samples using the following formula:

\[
\% \text{, cocoa (on a totally defatted basis) } = (T + C) \times 31
\]

Where -

- T: Theobromine content (%) in test sample determined in 7.2
- C: Caffeine content (%) in test sample determined in 7.2.

8. Calculation of fat-free cacao content

The content of cocoa (on a totally defatted basis) in test sample is calculated according to the following formula:

\[
\% \text{, cocoa (on a totally defatted basis) } = (T + C) \times 31
\]

Where-

- T: Theobromine content (%) in test sample determined in 7.2
- C: Caffeine content (%) in test sample determined in 7.2.

9. References

(3) AOAC 980.14