



Customs Laboratories
European Network

ILIADe 112:2023 | CLEN Method

**Determination of the cocoa content in food products by
quantification of theobromine and caffeine by HPLC**

Version 25 July 2023

This table shows the most important changes that have been made compared with the latest former version	
Date of the latest former version: 10 February 2022	
Section	Changes
8. Precision	repeatability and reproducibility confirmed for theobromine and updated for caffeine (following the CLEN 9 th Meursing proficiency test performed in 2021).

Determination of the cocoa content in food preparations by quantification of theobromine and caffeine by HPLC with UV detector.

1. Scope

The scope of this method is to measure the theobromine and caffeine content in food products. The cocoa content can be calculated from these two parameters. This method can be used for food samples where cocoa is the only source of theobromine and caffeine. If other theobromine and/or caffeine are present from other sources than cocoa, these quantities should not be taken into account in calculating the cocoa content.

For correct tariff classification of food products, it is necessary to differentiate food products containing cocoa versus products not containing cocoa, and/or to quantify the cocoa content (difference in classification between chapter 18 or another chapter).

2. Principle

Theobromine and caffeine are extracted from the sample, using water as solvent. After filtration, this water extract is analysed by HPLC with UV detection. The obtained peak areas of theobromine and caffeine can be recalculated into concentrations by use of an external calibration for both components. The cocoa content in food samples can be calculated from the measured theobromine and caffeine concentrations.

3. Reagents and materials

3.1 Theobromine (with purity > 98% for calibration)

3.2 Caffeine (with purity > 98% for calibration)

3.3 Solvent as mobile phase for HPLC (e.g. acetonitrile, water, methanol; HPLC grade)

3.4 Demineralized water to dissolve the samples

3.5 Calibration solutions prepared with theobromine (3.1) and caffeine (3.2). A typical range of the concentration of caffeine and theobromine in the calibration solutions are 1 to 200 mg/L (see 5.1 for detailed examples). Either a one point calibration or a multiple point calibration can be used. Attention has to be paid that the solubility of theobromine in water (around 300 mg/L) is not exceeded in calibration solutions.

Optional reagents, depending on the followed method

3.6 Petroleum ether (for defatting the sample)

All above reagents should be handled and stored according to their safety instructions.

4. Apparatus

- 4.1 Analytical balance with precision of 0.1 mg
- 4.2 HPLC with UV detector (able to measure at around 272 nm) or DAD detector
- 4.3 Volumetric flasks (e.g. 100 mL)
- 4.4 Pipettes
- 4.5 Filter (e.g. 0.20 μm) and syringe to filter the liquid
- 4.6 HPLC vials with caps
- 4.7 0.20 μm filter with appropriate syringe

Optional equipment, depending on the followed method:

- 4.8 Water bath (able to operate at temperatures of 60 – 100°C)
- 4.9 Centrifuge and centrifuge tubes
- 4.10 Magnetic stirrer with heating function
- 4.11 Erlenmeyer flasks
- 4.12 Stopper for Erlenmeyer flask
- 4.13 Boiling chips
- 4.14 Separatory funnel

5. Procedure

5.1 Sample preparation

General methodology. Weigh accurately on an analytical balance an amount of sample in a volumetric flask. The amount of sample depends upon the expected concentration of theobromine and caffeine (see the detailed example underneath for sample amount guidelines). The sample is dissolved in warm water and allowed to cool down to room temperature. Fill up the volumetric flask. This solution is filtered and analysed by HPLC with UV or DAD detector.

Detailed example 1. Homogenize the sample. Weigh accurately a representative sample amount in an Erlenmeyer flask. A guidance for the optimal sample amount is: milk chocolate 4.5 g, dark chocolate 1 g, chocolate biscuits: 10 g and cocoa powder: 0.6 g. Fill the Erlenmeyer flask with around 75 mL of demineralized water. Place the Erlenmeyer flask on a magnetic stirrer with heating function. Allow the solution to boil for at least 25 minutes while the solution is continuously stirred. After boiling, allow the solution to cool down to room temperature and transfer the solution quantitatively into a volumetric flask. The sample in the volumetric flask is diluted to the mark (100 mL) with water and homogenized. Pipet around 10 mL in a centrifugation tube and centrifuge for 10 minutes at 3000 g. Filter the supernatants (20 μm) and collect the filtrate in a HPLC vial.

Concentration range of the used calibration solutions: 2-20 mg theobromine / 100 mL and 0.2 – 2 mg caffeine / 100 mL.

Detailed example 2. Homogenize the sample. Weigh accurately a representative sample amount in a volumetric flask. A guidance for the optimal sample amount is: tea extract 0.5 g; protein rich samples 4.5 g; chocolate 1.5 g; and other samples between 0.5 and 2 g. Fill the volumetric flask with demineralized water at around 60°C for around 75%. Place the volumetric flask in a water bath at 100°C for 30 minutes. Shake vigorously after 15 minutes. Allow the solution to reach room temperature by placing the volumetric flask in cold water, fill the volumetric flask to the mark (100 mL), homogenize and filter. The filtrate is collected in a HPLC vial.

Concentration range of theobromine and caffeine calibration solutions: 10 – 200 mg/L

Detailed example 3. Accurately weigh, to 0.1 mg, approximately 0.6 g cocoa, 4.5 g milk chocolate, or 1.0 g chocolate liquor into weighed test tubes with Teflon-lined screw caps. To extract fat, shake twice with 30 mL petroleum ether, centrifuge at 2000 rpm for 10 minutes and carefully decant the solvent. Place the tubes in warm water bath in a fume hood to resolve solvent. Weigh the dried tubes and residue and calculate the weight of the residue. Quantitatively transfer contents with demineralized water and weighed 250 mL Erlenmeyer containing several boiling chips.

Add demineralized water to around 95 mL. Heat for minimum 25 minutes at 100°C. Cool to 20°C and add water so that final weight = weight flask with chips + weight defatted residue + 100.0 g. Ceil the flask with a stopper and shake to mix thoroughly. Transfer the mixture to a centrifuge tube, centrifuge for 5 minutes at 2000 rpm and filter a portion of the supernatants through a 0.45 µm membrane filter. Inject the filtrate into a chromatograph.

Calibration concentration of theobromine and caffeine: 50 mg/L.

Detailed example 4. Homogenize the sample. Weigh accurately a representative sample amount in a 100ml volumetric flask. Fill the volumetric flask with demineralized water at around 95°C for around 75%. Let it shake overnight. Next morning, fill the volumetric flask to the mark, homogenize and filter. Take an aliquot of 40ml of filtrate in a separatory funnel, add 30ml of petroleum ether and shake it. Allow the mixture to stand for some minutes and collect the lower phase, centrifuge and filter through a 0.2µl membrane filter. Collect the filtrate in a HPLC vial.

Concentration range of theobromine and caffeine in the calibration solutions: 1 – 10 mg/L.

5.2 HPLC measurement

The samples (calibration solution(s), control samples and samples) are measured using a HPLC with UV detector. Caffeine and theobromine have an absorption maximum at around 272 nm. Most HPLC software allow the input of sample weights (in g) and concentrations of calibration solutions (in mg/L) from which the theobromine and caffeine concentration in the sample are calculated automatically (weight %).

Possible HPLC conditions are listed in detail in Annex 1.

5.3 Quality control

The quality control can be performed by one (or a combination) of the following actions:

- Evaluation of measured peaks (symmetry, tailing, baseline separation, etc.)
- Evaluation of blank sample
- Evaluation of the calibration (the correlation coefficient of the calibration curve should be at least 0.995)
- Monitoring of the regression parameters in function of time and comparison of regression parameters between old and new calibration solutions
- Evaluation of duplicate measurements
- Measurement of control sample: use of a certified reference material is recommended to assess the quality of the entire procedure

6. Calculation

The corresponding peaks for theobromine and caffeine are detected and their peak areas are integrated by the HPLC software. The peak area can be recalculated into a concentration using the external calibrations of theobromine and caffeine according to:

$$\text{Peak area} = a \times \text{concentration (mg/L)} + b$$

(with a the slope of the calibration line and b the intercept of the calibration line)

Knowing the sample amount and the dilution volume, the concentrations can be recalculated into a theobromine and caffeine content (weight%) according to:

$$\text{Content (weight\%)} = \text{concentration (mg/L)} \times \text{dilution volume (L)} / \text{sample mass (mg)} \times 100$$

The cocoa powder content in the sample can be calculated (according to the general explanatory notes to chapter 19 in the combined nomenclature of the European union):

$$\text{Cocoa powder content (weight\%)} = [\text{theobromine content (weight\%)} + \text{caffeine content (weight\%)}] \times 31$$

Attention has to be paid if sources of caffeine and/or theobromine other than cocoa are present in the sample. The explanatory notes state: “*In the case of products containing caffeine or theobromine from sources other than cocoa, these additional amounts of caffeine or theobromine should not be taken into account in calculating the cocoa content*”.

7. Expression of the results

The cocoa content is given with maximum 2 significant digits, with one decimal.

8. Precision

The following prestatation characteristics should be feasible, based on the CLEN Meursing proficiency tests performed in 2015, 2018 and 2021 (9th Meursing test edition being the latest test performed in 2021) and results from volunteer laboratories, for the **limits of r and R**:

Theobromine

repeatability	15 %
Reproducibility	37 %

Caffeine

repeatability:	30 mg/kg abs for a caffeine concentration < 150 mg/kg
	20 % relative for a caffeine concentration > 150 mg/kg
Reproducibility:	68mg/kg abs for a caffeine concentration < 150 mg/kg
	45 % relative for a caffeine concentration > 150 mg/kg

LOD: 30 mg/kg for both theobromine and caffeine.

LOQ: 90 mg/kg for both theobromine and caffeine.

Annex

Detailed examples of possible method conditions. Alternative conditions can also be used as long as they give equivalent results.

Column	Details HPLC method																					
Zobax Eclipse plus 18C rapid resolution 4.6 x 100 mm	Mobile phase: acetonitrile/water with 0.1% tetrahydrofuran adjusted to pH 8 (10/90); isocratic method Flow rate: 0.8 ml/min Injection volume: 20 µL Detector: 272 nm																					
Prodigy 5 ODS-2, 4.6 mm x 25 cm	Mobile phase: methanol/water (30/70); isocratic method Flow rate: 2 ml/min Detector: 272 nm																					
Phenomenex – Kinetex 2.6 µm C18, 100 x 4.6 mm	Mobile phase: A: 0.2 mol/L KH ₂ PO ₄ in water, pH 2.35 B: Acetonitrile/water (76/24) <table style="margin-left: 40px;"> <tr> <td>0 min.</td> <td>100 % A</td> <td></td> </tr> <tr> <td>1 min.</td> <td>100 % A</td> <td></td> </tr> <tr> <td>4 min.</td> <td>93,5 % A</td> <td>6,5 % B</td> </tr> <tr> <td>7 min.</td> <td>62 % A</td> <td>38 % B</td> </tr> <tr> <td>8 min.</td> <td>62 % A</td> <td>38 % B</td> </tr> <tr> <td>8,5 min.</td> <td>100 % A</td> <td></td> </tr> <tr> <td>9,5 min.</td> <td>100 % A</td> <td></td> </tr> </table> Flow rate: 1.5 mL/min Detector: 273 nm Remark: longer run time may be needed for samples in order to avoid carry-over	0 min.	100 % A		1 min.	100 % A		4 min.	93,5 % A	6,5 % B	7 min.	62 % A	38 % B	8 min.	62 % A	38 % B	8,5 min.	100 % A		9,5 min.	100 % A	
0 min.	100 % A																					
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8 min.	62 % A	38 % B																				
8,5 min.	100 % A																					
9,5 min.	100 % A																					
µBondapak C18, 10 µm particle size, 4 mm id, 30 cm long	Mobile Phase: Methanol (chromatographic grade) – CH ₃ COOH - H ₂ O (20 + 1 + 79) Flow rate: 1 mL/min at 2000 psi nominal pressure Detector: 280 nm																					