SLOVENSKI STANDARD
SIST EN 12857:2000

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Živila - Določevanje ciklamata - Metoda tekočinske kromatografije visoke ločljivosti

Foodstuffs - Determination of cyclamate - High performance liquid chromatographic method

Lebensmittel - Bestimmung von Cyclamat - Hochleistungsflüssigchromatographisches Verfahren

Produits alimentaires - Dosage du cyclamate - Méthode par chromatographie liquide haute performance

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ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

SIST EN 12857:2000 **en**

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English version

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Central Secretariat: rue de Stassart, 36 B-1050 Brussels

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Contents

Foreword

This European Standard has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 1999, and conflicting national standards shall be withdrawn at the latest by October 1999.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This draft European Standard specifies a high performance liquid chromatographic (HPLC) method for the determination of sodium cyclamate in foodstuffs [1], [2], [3]. The method has been validated in an inter-laboratory test according to ISO 5725 : 1986 [4] which has been carried out with lemonade, orange juice beverage, fruit yogurt and spray cream.

In addition to the matrices investigated in the inter-laboratory test, experiences have shown that the method is also applicable to various foodstuffs, such as gherkins, canned morello cherries, pineapple, orange nectar, apricot jam, blackberry jam, cherry nectar, hard candy, mixed fruit yogurt, strawberry yogurt, fruit quark, rice-pudding-apple-raisins, chocolate custard powder, cream dessert vanilla powder, vanilla ice, passion fruit-orange ice cream, [2], [3].

2 Normative references

This draft European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this draft European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

EN ISO 3696 Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)

3 Principle

Sodium cyclamate is extracted from the sample with water, converted to N, N-dichlorocyclohexylamine and determined by HPLC on a reversed-phase column using UV detection at a wavelength of 314 nm.

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 as defined in EN ISO 3696. When preparing solutions, the purity of the substances shall be taken into account.

4.1 Methanol, for HPLC

4.2 *n*-Heptane, for HPLC

4.3 Light petroleum, having a boiling range from 40 °C to 60 °C

4.4 Sodium sulfate, anhydrous. If necessary, wash with *n*-heptane to remove lipophilic contaminants

4.5 Sodium carbonate solution, ρ (Na_2CO_3) = 50 g/l [1])

4.6 Sodium hypochlorite solution (1,7 % active chlorine)

Dilute commercially available sodium hypochlorite solution containing more than 1,7 % active chlorine with water to get a mass fraction of 1,7 % of active chlorine. Check the content of active chlorine of the sodium hypochlorite solution regularly, e.g. using the procedure as described in annex A.

4.7 Sulfuric acid, w (H_2SO_4) = 50 % [2])

4.8 Sodium cyclamate standard solutions

Weigh, to the nearest 0,1 mg, about 898 mg of sodium cyclamate in a 200 ml volumetric flask and dilute to the mark with water, ρ (cyclohexylsulfamic acid) = 800 mg/200 ml[3]). Pipette 0,25 ml, 1 ml, 2,5 ml, 5 ml, 10 ml and 20 ml of this solution in 100 ml volumetric flasks and dilute to the mark with water. The cyclohexylsulfamic acid concentration of these solutions is 10 mg/l, 40 mg/l, 100 mg/l, 200 mg/l, 400 mg/l and 800 mg/l.

4.9 Carrez solution No 1

Dissolve 15 g of potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6] \cdot 3\,H_2O$) with a mass fraction of at least 99 % in water and dilute to 100 ml.

4.10 Carrez solution No 2

Dissolve 30 g zinc sulfate ($ZnSO_4 \cdot 7\,H_2O$) with a mass fraction of at least 99,5 % in water and dilute to 100 ml.

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[1]) ρ is the mass concentration

[2]) w is the mass fraction

[3]) The conversion factor from sodium cyclamate to cyclohexylsulfamic acid is 0,8909

4.11 Mobile phase

Mix 80 parts per volume methanol (4.1) with 20 parts per volume water, filter through suitable membrane filters, e.g. of pore size 0,45 µm and degas, e.g. for 5 min in an ultrasonic bath.

If neccessary, the proportion of methanol to water in the mobile phase may have to be modified slightly, to achieve adequate results with the column used.

4.12 Conditioning solution for HPLC, EDTA solution, ρ (EDTA) = 10 g/l, filter through suitable membrane filters, e.g. of pore size 0,45 µm and degas, e.g. for 5 min in an ultrasonic bath.

4.13 Cellulose, powdered, with a mass fraction of at least 99,9 %, acid washed

5 Apparatus and equipment

Usual laboratory apparatus and, in particular, the following

5.1 HPLC apparatus, comprising the following

5.1.1 High performance liquid chromatograph, consisting of a pump, a sample injector, an ultraviolet (UV) spectrometer (capable of operating at a wavelength of 314 nm, preferably a diode array detector), with a recorder and/or integrator which allows the measurement of peak heights and peak areas.

5.1.2 Analytical reversed phase separating column, e.g. with

- a RP C 18 stationary phase of 5 µm,
- a length of 250 mm,
- internal diameter of 4 mm,
- a guard column, RP C 18 (optional, but recommended especially for all solid sample materials).

Performance criterion for suitable analytical columns is the baseline resolution of the cyclamate peak.

Whenever interferences are identified with a diode array detector or by measurement at a second wavelength, an alternative chromatographic condition shall be chosen for the determination of cyclamate.

5.2 Ultrasonic bath

5.3 Membrane filter, of suitable pore size, e.g. 0,45 µm

5.4 Filtration unit with filter holder for membrane filter for filtering and degassing mobile phase (4.11) and conditioning solution (4.12)

5.5 Homogenizer

5.6 Water bath, capable of being maintained at 60 °C

5.7 Centrifuge, capable of producing a centrifugal acceleration of at least 1400 *g* at the base of the centrifuge tubes (5.8)

5.8 Centrifuge tubes, preferably made of glass, of suitable capacity, e.g of 50 ml, sealable

5.9 Separating funnels, of suitable capacity, e.g. of 50 ml and 100 ml

5.10 Fluted filter paper, medium fast, qualitative

5.11 Phase separation filters, (optional)

6 Procedure

6.1 Preparation of the sample solutions

6.1.1 Liquid products and products giving clear solutions (e.g. clear fruit juices, filtered gherkin brine, hard candy)

Dilute liquid products, filtered if neccessary, with water to give a cyclamate content of approximately 400 mg/l or take liquid products directly for derivatization. Dissolve solid products in water to give a clear solution having a cyclamate content of approximately 400 mg/l.

6.1.2 Semi solid samples, (e.g. dairy products, desserts, spray cream, cloudy juices, jams, marmalades)

Homogenize the sample thoroughly for 1 min. Weigh, to the nearest 1 mg, about 15 g of the sample into a 100 ml volumetric flask, add 80 ml of water and place the flask for approximately 10 min in an ultrasonic bath (5.2). Any other mass of the sample may be chosen yielding a cyclamate content of not more than 400 mg/l in the 100 ml volumetric flask. Add 1 ml to 2 ml of Carrez solution No 1 (4.9), mix, and add the same volume of Carrez solution No 2 (4.10). Mix, dilute to the mark with water. Filter through a fluted filter paper, discarding the first 10 ml of the filtrate.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at a centrifugal acceleration of at least 1400 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Take 20 ml of this solution for derivatization (see 6.2).

6.1.3 Chocolate and related products

Weigh, to the nearest 1 mg, about 15 g of the sample into a centrifuge tube (5.8) and stand the centrifuge tube in a water bath at 60 °C until the sample melts completely. Carefully and slowly add 25 ml of light petroleum (4.3), mix thoroughly, seal the centrifuge tube and place it in an ultrasonic bath for 30 s and mix again. Centrifuge the sealed centrifuge tubes for 10 min at at least 1400 *g*. Decant the light petroleum layer and repeat the extraction with 25 ml of light petroleum, again decanting the light petroleum layer. Evaporate remaining light petroleum by placing the centrifuge tube for 15 min in a water bath at 60 °C and mixing. Add 30 ml of water and mix thoroughly. Stand the centrifuge tube for 5 min in the ultrasonic bath. Transfer the solution with approximately 40 ml of water quantitatively into a 100 ml volumetric flask, add 1 ml of Carrez solution No 1 (4.9), mix, add 1 ml of Carrez solution No 2 (4.10) and mix thoroughly. Bring the solution to a temperature of 20 °C, dilute to the mark with water. Filter through a fluted filter paper, discarding the first 10 ml of the filtrate.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at a centrifugal acceleration of at least 1400 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Take 20 ml of this solution for derivatization (see 6.2).

6.1.4 Fat emulsions and products containing those (e.g. mayonnaise)

Homogenize the sample thoroughly. Weigh, to the nearest 1 mg, about 15 g of homogenized mayonnaise into the centrifuge tube (5.8). Add 2,5 g of powdered cellulose (4.13) and mix. Add 25 ml of light petroleum (4.3) and mix. Seal the centrifuge tube and place it in an ultrasonic bath for 30 s and mix again. Centrifuge for 10 min at a centrifugal acceleration of at least 1400 *g*.

Decant the light petroleum layer and repeat extraction with 25 ml of light petroleum again decanting the light petroleum layer. Evaporate remaining light petroleum by placing the centrifuge tube for 15 min in a water bath at 60 °C and mixing. Add 40 ml water and thoroughly mix. Sonicate the centrifuge tube for 10 min in the ultrasonic bath.

Transfer the solution with approximately 30 ml water quantitatively into a 100 ml volumetric flask. Bring the solution to a temperature of 20 °C, add 1 ml of Carrez solution No 1 (4.9), mix, add 1 ml of Carrez solution No 2 (4.10), mix thoroughly and dilute to the mark with water. Filter through a fluted filter paper, discarding the first 10 ml of the filtrate.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approximately 3 g, it is advisable to centrifuge the clarified sample mixture for 10 min at a centrifugal acceleration of at least 1400 *g* before filtering it quantitatively into the 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatants in the 100 ml volumetric flask and then dilute the solution to the mark with water. This procedure may also be followed when the amount of insoluble matter is less than 3 g.

Take 20 ml of this solution for derivatization (see 6.2)

6.2 Derivatization

WARNING: Due to the generation of chlorine gas, carry out the derivatization under a well working fume hood.

To obtain the sample test solution or standard test solution, pipette 20 ml of the sample solutions obtained in 6.1.1 to 6.1.4 or the standard solutions (4.8) into a separating funnel (5.9), add 1 ml of sulfuric acid (4.7), 10,0 ml of

n-heptane (4.2) and 2,5 ml of sodium hypochlorite solution (4.6), and shake vigorously for 1 min. Allow the phases to separate. Discard the lower, aqueous phase. Intractable emulsion is treated like the *n*-heptane layer. Wash the *n*-heptane layer with 25 ml of sodium carbonate solution (4.5) by vigorously shaking for 30 s. Discard the lower, aqueous phase. If the phases separate clearly, dry the *n*-heptane layer with 1 g of sodium sulfate (4.4) and filter through a fluted filter paper. If emulsions cannot be broken, dry the *n*-heptane phase with approximately 7 g of sodium sulfate and filter through a fluted filter paper, or use a phase separation filter (5.11).

The derivated solutions are stable for 24 h at +4 °C.

6.3 Identification

Identify cyclamate in the sample test solution by comparing the retention time of the analyte in the sample solution with that of the standard substance, or by simultaneous injection of the standard solution and the sample test solution, or by adding the standard solution to the sample test solution and recording an absorption curve in the relevant wavelength range.

Inject equal volumes of the sample test and standard test solutions. Intervals between successive injections of the standard solutions should be not less than 15 min. To minimize the risk that substances eluted from earlier injections will be confused with components from subsequent samples, successive injections of the sample test solutions should be made at intervals sufficiently long (e.g. 30 min).

If the column specified in 5.1.2 is used, it has been found satisfactory to adopt the following chromatographic conditions:

Mobile phase:	as described in 4.11
Flow rate:	1,0 ml/min
Injection volume:	20 µl
Detection (UV):	314 nm

For conditioning, each day before starting the analyses, rinse the column for 10 min with water, 30 min with conditioning solution (4.12) and again 10 min with water.

A specimen chromatogram is given for information in Annex B.

6.4 Determination

For the determination by the external standard method, integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard substance with the nearest peak area/height, or use a calibration graph.

To prepare the calibration graph, inject a suitable amount of standard solutions of appropriate mass concentrations. Plot the peak heights or peak areas of the sodium cyclamate standard solutions against the corresponding cyclohexylsulfamic acid mass concentrations in milligrams per litre. Check the linearity of the calibration graph.

Alternatively, the calibration may also be evaluated mathematically by the regression. Check the linearity of the regression graph.

7 Calculation

7.1 External standard method

Calculate the mass fraction, *w*, expressed in milligrams per kilogram, or the mass concentration, ρ, in milligrams per litre, of cyclohexylsulfamic acid using equation (1):

$$w \text{ or } \rho = \frac{A_1 \cdot V_1 \cdot m_1 \cdot F}{A_2 \cdot V_2 \cdot m_0} \cdot 1000 \qquad (1)$$

where:

A_1 is the peak area of cyclamate derivate obtained with the sample test solution;
A_2 is the peak area of cyclamate derivate obtained with the standard test solution;
V_1 is the volume of the sample solution, in millilitres (here: 100 ml);
V_2 is the volume of the standard solution, in millilitres (here: 100 ml);
m_1 is the mass of cyclamate in the standard solution (V_2), in milligrams;
m_0 is the mass of the test portion, in grams or milliliters;
F is the dilution factor taking account of any other dilution not described here in detail that is necessary during analysis.

7.2 Calibration graph

Calculate the mass fraction, w, expressed in milligrams per kilogram, or the mass concentration, ρ, in milligrams per litre, of cyclohexylsulfamic acid using equation (2):

$$w \text{ or } \rho = \frac{\rho_{cs} \cdot V_1 \cdot F}{m_0} \qquad (2)$$

where:

ρ_{cs} is the mass concentration of cyclohexylsulfamic acid in the sample test solution, read off from the calibration or regression graph, in milligrams per litre;

V_1, m_0, F see equation 1

7.3 Expression of results

Report the result without a decimal place.

NOTE: Conversion factor from sodium cyclamate to cyclohexane sulfamic acid = 0,8906

8 Precision

8.1 General

Details of the inter-laboratory test according to ISO 5725:1986 [4] of the precision of the method are summarized in annex C. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than given in annex C.

8.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases.

The values are:

lemonade	$\bar{x}$ = 435,9 mg/l	r = 16,7 mg/l
orange juice beverage	$\bar{x}$ = 178,3 mg/l	r = 15,4 mg/l
spray cream	$\bar{x}$ = 280,9 mg/kg	r = 26,0 mg/kg
fruit yogurt	$\bar{x}$ = 647,6 mg/kg	r = 42,4 mg/kg

8.3 Reproducibility

The absolute difference between two single test results on identical test material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases.

The values are:

lemonade	$\bar{x}$ = 435,9 mg/l	R = 38,1 mg/l
orange juice beverage	$\bar{x}$ = 178,3 mg/l	R = 24,4 mg/l
spray cream	$\bar{x}$ = 280,9 mg/kg	R = 49,7 mg/kg
fruit yogurt	$\bar{x}$ = 647,6 mg/kg	R = 168,4 mg/kg

9 Test report

The test report shall contain at least the following data:

- all information necessary for the identification of the sample;
- a reference to this draft European Standard or to the method used;
- date and type of sampling procedure (if known);
- date of receipt;
- date of test;
- the results and the units in which the results have been expressed;
- any particular points observed in the course of the test;
- any operations not specified in the method or regarded as optional which might have affected the results.