BS EN 12014-2:2017

BSI Standards Publication

Foodstuffs - Determination of nitrate and/or nitrite content

Part 2: HPLC/IC method for the determination of nitrate content of vegetables and vegetable products

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National foreword

This British Standard is the UK implementation of EN 12014-2:2017. It supersedes BS EN 12014-2:1997, which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Foodstuffs - Determination of nitrate and/or nitrite content - Part 2: HPLC/IC method for the determination of nitrate content of vegetables and vegetable products

Produits alimentaires - Détermination de la teneur en nitrates et/ou en nitrites - Partie 2 : Méthode de détermination par CLHP/CI de la teneur en nitrates des légumes et des produits à base de légumes

Lebensmittel - Bestimmung des Nitrat- und/oder Nitritgehaltes - Teil 2: HPLC/IC-Verfahren für die Bestimmung des Nitratgehaltes in Gemüse und Gemüseerzeugnissen

This European Standard was approved by CEN on 15 October 2017.

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European foreword

This document (EN 12014-2:2017) 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of an identical text or by endorsement, at the latest by June 2018, and conflicting national standards shall be withdrawn at the latest by June 2018.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 12014-2:1997.

The following changes have been made to the former edition:

a) the scope has been reduced from 50 mg/kg nitrate to 25 mg/kg nitrate and the upper limit has been deleted;

b) the purification method 1 and 2 used in the preparation of sample test solutions has been deleted;

c) a new matrix (iceberg lettuce) has been verified in an interlaboratory test;

d) update of the HPLC/IC-conditions and chromatograms in Annex A;

e) the procedure has been extensively revalidated and precision data in Annex B have been revised;

f) editorially revised.

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Introduction

The method described in this standard has been developed and validated for investigations regarding the European legislation for nitrate in vegetables and vegetable products. Laboratory experience has shown that this analytical method is also suitable for the determination of nitrite in other matrices; however, this has not been validated in the interlaboratory test scheme cited here.

1 Scope

This European Standard specifies a high-performance liquid chromatographic (HPLC) and an ion chromatographic (IC) method for determination of the nitrate level in vegetables and vegetable products. This method is applicable for samples with a content of 25 mg/kg or greater.

It has been validated on naturally contaminated and spiked samples as beetroot juice with nitrate mass fractions of 194 mg/kg and 691 mg/kg, pureed carrots with nitrate mass fractions of 26 mg/kg and 222 mg/kg and with iceberg lettuce with nitrate mass fractions of 623 mg/kg and 3 542 mg/kg.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Principle

The nitrate is extracted from the foodstuff with hot water. The determination is performed either by reverse-phase HPLC and UV detection, or by IC and conductivity or UV detection.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified. Water shall be nitrate-free and shall comply with grade 1 of ISO 3696.

4.1 Potassium nitrate.

4.2 Sodium nitrate.

4.3 Sulfuric acid, 96 % (ρ = 1,84 g/ml[1)]).

4.4 Regenerator for suppressor, e.g. sulfuric acid (c = 0,0125 mol/l[2)]).

Carefully pipette 20 ml of sulfuric acid (4.3) into a 1 000 ml volumetric flask containing 800 ml of water. Mix the solution, dilute to the mark with water and mix again. Transfer 33 ml of this solution to a 1 000 ml volumetric flask containing 500 ml of water. Dilute to the mark with water and mix.

Alternatively, use a method with electrolytic suppressor.

4.5 Nitrate stock solution, ρ = 1 000 mg/l (expressed as ion NO_3^-).

Weigh 1,630 7 g of potassium nitrate (4.1) to the nearest 0,1 mg and dissolve in water in a 1 000 ml volumetric flask.

Alternatively, 1,370 9 g of sodium nitrate (4.2) can be used. If required, 15 ml of sulfuric acid (4.3) can be added as a preservative. Mix the solution, dilute to the mark with water and mix again. This solution is stable for at least two months if stored at +4 °C in the dark (refrigerator).

Alternatively, commercially available standard solutions can also be used.

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1) ρ = mass concentration

2) c = substance concentration

4.6 Nitrate standard solutions.

Prepare suitable dilutions from the nitrate stock solution (4.5), preferably in the range of 1 mg/l to 50 mg/l. Pipette an appropriate volume of nitrate stock solution into a 100 ml volumetric flask, dilute to the mark with water, mix and filter through a membrane filter (5.4), if required.

4.7 Mobile phase, for the HPLC.

Examples of suitable eluents are given in Annex C.

4.8 Mobile phase, for the IC.

Examples of suitable eluents are given in Annex D.

5 Apparatus and equipment

Usual laboratory equipment and, in particular, the following:

5.1 Commercially available laboratory mixer, e.g. a laboratory cutter.

5.2 Homogenizer.

5.3 Fluted filter paper (nitrogen-deficient).

5.4 Membrane filter, for aqueous solutions, with a pore size of 0,45 µm (e.g. regenerated cellulose).

5.5 Filter holder for membrane filter, with suitable syringe.

5.6 HPLC equipment.

5.6.1 High-performance liquid chromatograph, consisting of an eluent reservoir, a pump, a sample applicator, a UV detector adjustable from 190 nm to 800 nm and an evaluation unit.

5.6.2 Analytical (reversed-phase) separating column.

Examples of suitable separating columns are given in Annex C.

Use a pre-column with filling material of the same type to protect the analytical separating column.

5.7 IC apparatus.

5.7.1 Ion-exchange chromatography unit, consisting of an eluent reservoir, a pump, a pulsation damper, a dialysis unit (if required), a suppressor (if required), a sample applicator with a sample loop, a conductivity detector or UV detector adjustable from 190 nm to 800 nm and an evaluation unit.

5.7.2 Analytical separating column.

Examples of suitable separating columns are given in Annex D.

Use a pre-column with filling material of the same type to protect the analytical separating column.

6 Procedure

6.1 Sample preparation

6.1.1 Liquid samples, e.g. vegetable juice

Vigorously shake a representative sample until it is thoroughly mixed. If required, filter the sample through a fluted filter paper (5.3) or, if necessary, predilute it with water to achieve a nitrate concentration of approximately 25 mg/l in the solution.

6.1.2 Solid samples, e.g. leaf vegetable

Take a representative sample and remove adhering animals, soil or dirty particles. If required, remove any inedible or damaged external leaves. Shred the sample prepared in this way, e.g. in a laboratory cutter (5.1) and thoroughly homogenize it using a homogenizer (5.2). Analyse the sample immediately after homogenization.

The requirements of the European legislation shall be taken into account, e.g. [1], [2] [3)]. If immediate testing of the sample homogenate is not possible, immediately freeze the sample. For analysis, thaw the sample as gently as possible (e.g. overnight in the refrigerator).

If it is not possible to homogenize a fresh produced sample within 24 h, the whole sample should be frozen and analysed within a maximum of 6 weeks.

6.1.3 Pasty samples, e.g. mashed vegetables

Repeatedly stir a representative sample, e.g. by using a homogenizer until it is thoroughly mixed.

6.2 Hot water extraction of samples (solid and pasty samples)

From the sample prepared in accordance with 6.1.2 or 6.1.3, weigh approximately 10 g of material to the nearest of 1 mg into a 500 ml conical flask. Add approximately 400 ml of hot water (approximately 80 °C) and place the conical flask into a boiling water bath. When the water bath has reached boiling point temperature again, keep the flask there for 15 min. Cool to room temperature, transfer the solution into a 500 ml volumetric flask, dilute to the mark, shake thoroughly and filter the contents through a fluted filter paper (5.3).

For sample material with low nitrate content, make appropriate adjustments, if necessary, to the initial test portions and volumetric ratios. When the test portion is increased or decreased (e.g. dried products) it is recommended to check the effect of this modification on the method performance.

6.3 Preparation of sample test solutions

If necessary, filter the sample solutions (6.1.1 and 6.2) through a suitable membrane filter (5.4). At least a minimum of 15 ml of test solution (e.g. starch containing samples) is required. Discard the first few millilitres of sample (to avoid possible contamination). The filtrate is dialysed or directly injected. Obtain at least one reagent blank value in parallel.

6.4 Preparation of the calibration curve

To plot a calibration curve, prepare a series of standard solutions (4.6) of suitable concentrations (generally five different concentration levels and one blank value). Inject equal volumes of the sample solution and standard solutions as described in 7.1. Check the calibration curve using freshly prepared standard solutions.

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3) For example, the provisions of Commission Regulation (EC) No. 1882/2006 regarding sample preparation (in the version of 2015) stipulate that samples shall not be washed.

6.5 Determination

6.5.1 High-performance liquid chromatography

Examples of HPLC conditions which have proven useful in the interlaboratory test are given in Annex C.

6.5.2 Ion chromatography

Examples of IC conditions which have proven useful in the interlaboratory test are given in Annex D.

7 Evaluation

7.1 Identification and quantitative determination

Inject a suitable volume (see Table C.1 and D.1) of the sample test solution (6.3) and standard solution (4.6) into the HPLC/IC-system. Examples of HPLC/IC chromatograms are given in Annex A. Identify the nitrate peak by comparing the retention times for standard solution and sample test solution.

Perform the quantitative determination in mg/kg or mg/l using a calibration function produced on the basis of the peak area or the peak height.

If the peak area/peak height of the measured sample test solution falls outside the range of the calibration curve, dilute the sample test solution with water and repeat the analysis.

The injected volumes of sample test solutions and standard solutions shall be identical.

7.2 Calculation

Calculate the mass fraction of nitrate w in mg/kg or the mass concentration ρ in mg/l of the sample using Formula (1):

$$w \text{ or } \rho = \frac{x \cdot F \cdot 1000 \cdot V}{m} \quad (1)$$

where

x is the nitrate concentration of the sample test solution read from the calibration curve minus the reagent blank value, in mg/l;

F is the dilution factor resulting from any further dilution steps;

m is the used sample amount, in g or ml;

V is the volume of the extraction solution (6.2), in l, here 0,5 l.

Report the result in mg/kg or mg/l with no decimal places, according to current legislation.

7.3 Precision

7.3.1 General

Details of an interlaboratory test for the precision of the method for beetroot juice, carrot purée and iceberg lettuce are given in Table B.1. The values derived from the interlaboratory test are possibly not applicable to concentration ranges and/or matrices other than those given in Annex B.

7.3.2 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:

Beetroot juice, naturally contaminated	$\bar{x}$ = 194 mg/kg	*r* = 14 mg/kg
Beetroot juice, spiked	$\bar{x}$ = 691 mg/kg	*r* = 17 mg/kg
Puréed carrots, naturally contaminated	$\bar{x}$ = 26 mg/kg	*r* = 3,3 mg/kg
Puréed carrots, spiked	$\bar{x}$ = 222 mg/kg	*r* = 12 mg/kg
Iceberg lettuce, naturally contaminated	$\bar{x}$ = 623 mg/kg	*r* = 39 mg/kg
Iceberg lettuce, spiked	$\bar{x}$ = 3 542 mg/kg	*r* = 105 mg/kg

7.3.3 Reproducibility

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

The values are:

Beetroot juice, naturally contaminated	$\bar{x}$ = 194 mg/kg	*R* = 51 mg/kg
Beetroot juice, spiked	$\bar{x}$ = 691 mg/kg	*R* = 32 mg/kg
Puréed carrots, naturally contaminated	$\bar{x}$ = 26 mg/kg	*R* = 12 mg/kg
Puréed carrots, spiked	$\bar{x}$ = 222 mg/kg	*R* = 71 mg/kg
Iceberg lettuce, naturally contaminated	$\bar{x}$ = 623 mg/kg	*R* = 122 mg/kg
Iceberg lettuce, spiked	$\bar{x}$ = 3 542 mg/kg	*R* = 876 mg/kg

8 Test report

The test report should consider the requirements as in EN ISO/IEC 17025 and shall contain as a minimum the following data:

a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this European Standard;

c) date and type of sampling procedure (if known);

d) date of the sample receipt;

e) date of testing;

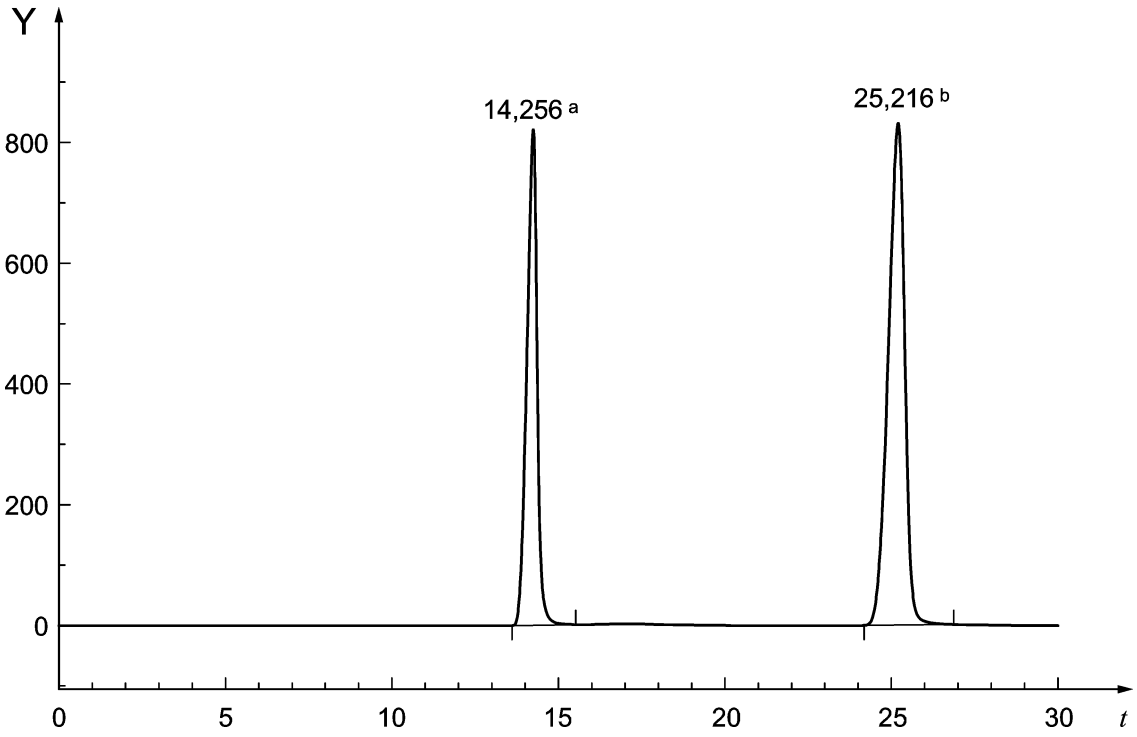
f) test results and units in which they have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

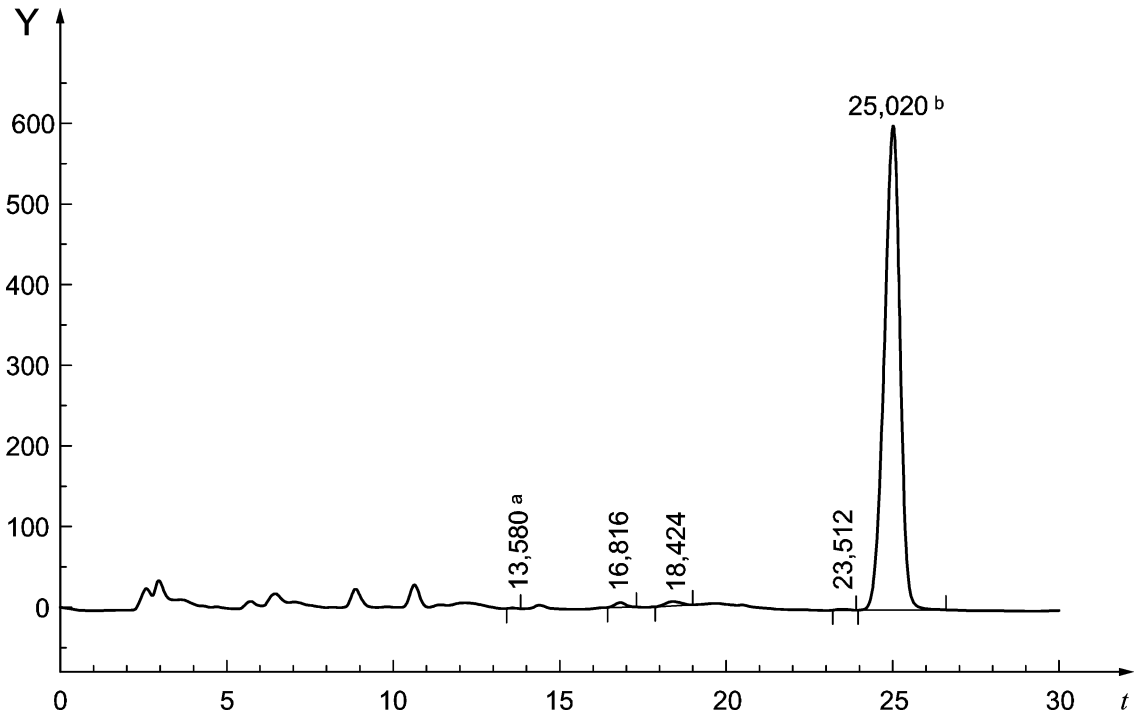
Examples of HPLC/IC chromatograms



Key

Y	intensity, in mAU	a	nitrite
t	time in min	b	nitrate

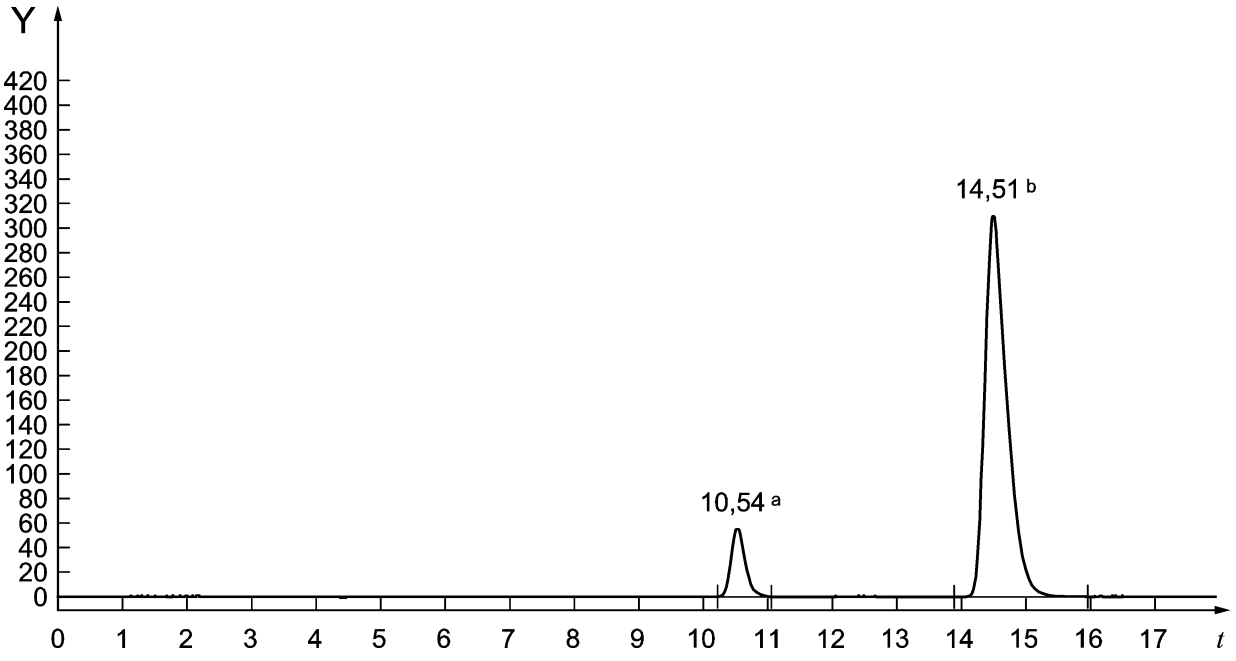
Figure A.1 — Chromatogram of a standard solution (nitrate: 76,6 mg/l; nitrite: 60,2 mg/l) using the HPLC conditions described in Table C.1, Column b



Key

Y	intensity, in mAU	a	nitrite
t	time in min	b	nitrate

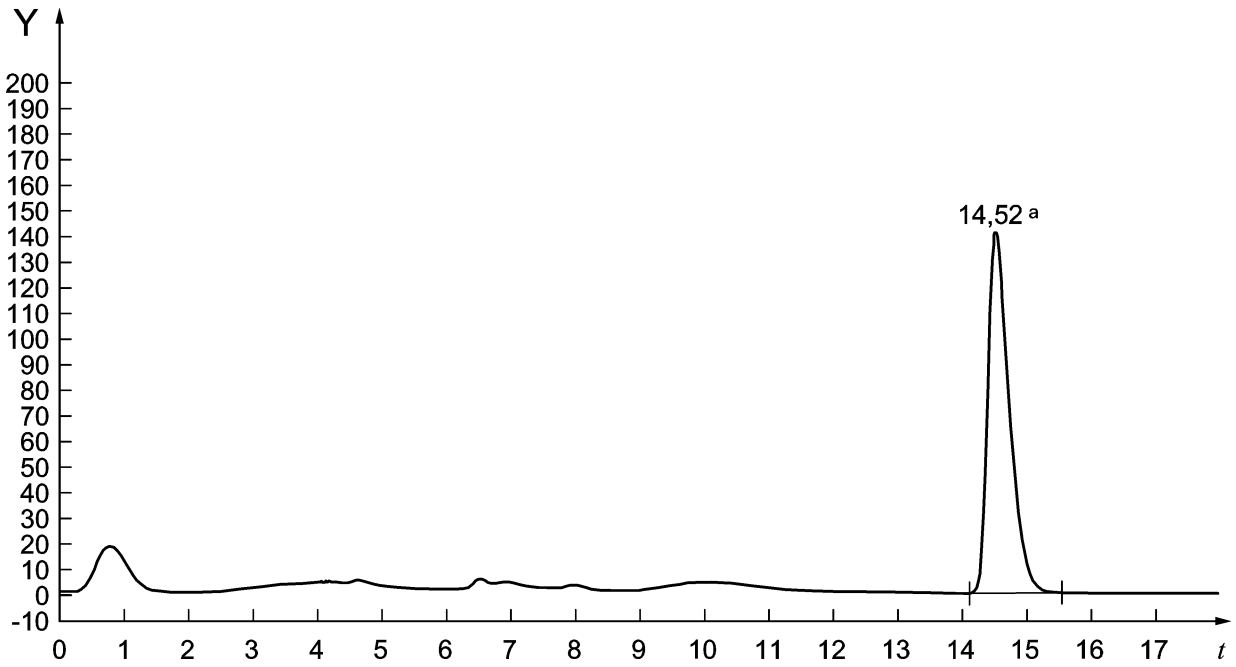
Figure A.2 — Chromatogram of rocket lettuce using the HPLC conditions described in Table C.1, Column b



Key

Y	intensity, in mAU	a	nitrite
t	time in min	b	nitrate

Figure A.3 — Chromatogram of a standard solution (nitrate: 10 mg/l; nitrite: 1 mg/l) using the IC conditions described in Table D.1, Column c



Key

Y intensity, in mAU a nitrate

t time in min

Figure A.4 — Chromatogram of a sample solution (cos lettuce, nitrate 840 mg/kg) using the IC conditions described in Table D.1, Column c

Annex B
(informative)

Precision data

This method has been developed by the § 64 LFGB working group “Nitrate/Nitrite” of the 'Bundesamt für Verbraucherschutz und Lebensmittelsicherheit' for the implementation of § 64 LFGB (German Foodstuffs and Feed Act) and validated by an interlaboratory test with 12 participating laboratories in accordance with ISO 5725.

The nitrate content was determined in three different samples (beetroot juice, carrot purée and iceberg lettuce). A total of six laboratories performed measurements using HPLC and a further six using IC.

The reproducibility standard deviation for all samples was less than double the associated relative Horwitz standard deviation (HorRat < 2), so that the method fulfils this performance criterion in accordance with Commission Regulation (EC) No. 1882/2006 [2].

Table B.1 — Results from the statistical evaluation of the ring trial

Statistical parameter	**Beetroot juice** nc [a]	**Beetroot juice** spiked + 500 mg/kg	**Carrot purée** nc	**Carrot purée** spiked + 200 mg/kg	**Iceberg lettuce** nc	**Iceberg lettuce** spiked + 3 000 mg/kg
Year of the interlaboratory test	2013	2013	2013	2013	2013	2013
Number of participating laboratories	12	12	12	12	12	12
Number of laboratories retained after eliminating outliers	12	11	12	12	11	12
Number of outlier laboratories	0	1	0	0	1	0
Mean value, mg/kg ± confidence interval	194 ± 9,9	691 ± 5,0	26 ± 2,2	222 ± 14	623 ± 24	3 542 ± 156
Reproducibility standard deviation SD s_R, mg/kg	18	11	4,3	25	43	313
Relative reproducibility standard deviation $s_{R,rel}$, %	9,4	1,6	17	11	7,0	8,8
Reproducibility limit R, mg/kg	51	32	12	71	122	876
Relative Reproducibility limit R_{rel},%	26	4,6	46	32	20	25
Intermediate SD s_I, mg/kg	7,7	10	2,9	9,0	21	221
Relative intermediate SD $s_{I,rel}$,	4,0	1,5	11	4,1	3,4	6,2

Statistical parameter	Beetroot juice nc [a]	Beetroot juice spiked + 500 mg/kg	Carrot purée nc	Carrot purée spiked + 200 mg/kg	Iceberg lettuce nc	Iceberg lettuce spiked + 3 000 mg/kg
%						
Intermediate limit I (mg/kg)	22	28	8,2	25	59	618
Relative intermediate limit I_{rel}, %	11	4,1	32	11	9,4	17
Repeatability SD s_r, mg/kg	5,1	6,1	1,2	4,3	14	38
Relative repeatability SD $s_{r,rel}$, %	2,6	0,88	4,6	1,9	2,2	1,1
Repeatability limit r, mg/kg	14	17	3,3	12	39	105
Relative repeatability limit r_{rel}, %	7,3	2,5	13	5,4	6,2	3,0
Rel. Horwitz SD, %	7,2	6,0	10	7,1	6,1	4,7
HorRat	1,3	0,27	1,7	1,6	1,2	1,9
[a] nc: naturally contaminated						

The method was checked based on a graduated nested interlaboratory test design; every sample was prepared and analysed twice on one day. A single determination was conducted after one to two weeks. The variability between the test days ascertained on this basis is identified by the intermediate standard deviation (s_I) [3]. This provides additional information concerning the precision of the measuring method.

In order to determine the recovery rate within the framework of this interlaboratory test, the three sample materials were additionally spiked with nitrate solution and analysed. For this, to each unspiked sample material (6.1.1 to 6.1.3), an appropriate quantity of nitrate standard solution was added (beetroot juice: 500 mg/kg, carrot purée: 200 mg/kg, iceberg lettuce: 3 000 mg/kg).

As a result, the mean recovery rates (see Table B.2) were within the recommended range (60 % to 120 % for nitrate levels < 500 mg/kg, or 90 % to 110 % for nitrate levels ≥ 500 mg/kg) in accordance with Commission Regulation (EC) No. 1882/2006 [2].

Table B.2 — Recovery results

Statistical parameter	Beetroot juice	Carrot purée	Iceberg lettuce
Recovery (R)	99 %	100 %	97 %
Interlaboratory precision SD for laboratory-specific R	4 %	7 %	7 %

Annex C
(informative)

Examples of suitable HPLC conditions

Table C.1 — Examples of suitable HPLC conditions

HPLC conditions	a	b	c
Column	Nucleosil® 120–5 C18 / Phenomenex® Luna C18 a	Purospher® STAR 100 C18	HyperClone™ ODS (C18) 120 Phenomenex®
Column dimension in mm	(100 to 125) × 4	(125 to 250) × 3	100 × 4
Particle size	5 µm	5 µm	3 µm
Precolumn	As separation column	As separation column	As separation column
Mobile phase	Acetonitrile/ n-octylamine pH 4,0	Tetrabutylammonium hydrogen sulfate pH 5,0	Tetrabutylphosphonium chloride/ Na_2HPO_4 / acetonitrile
Flow rate in ml/min	0,5 to 1	0,2 to 0,7	0,6
Injection volume in µl	5 to 20	10 to 50	10
Column temperature in °C	20 to 40	20 to 30	40
Detection wavelength in nm	205 to 210	205 to 210	205 to 210
a Nucleosil® 120–5 C18, Phenomenex® Luna C18, Purospher® STAR 100 C18 and HyperClone™ ODS (C18) 120 Phenomenex® are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not consitute an endorsement by CEN.			

Production of mobile phase:

a) Add 70 ml of acetonitrile and 1,66 ml of n-octylamine to about 800 ml of water. Adjust the pH to 4,0 with o-phosphoric acid (w = 85 %). Transfer the solution to a 1 000 ml volumetric flask and dilute to the mark with double-distilled water.

b) Dissolve 1 g of tetrabutylammonium hydrogen sulfate in 800 ml of double-distilled water and adjust the pH to 5,0 with $K_2HPO_4 \times 3\ H_2O$ (c = 20 mmol/l ≈ 4,56 g/l). Transfer the solution to a 1 000 ml volumetric flask and dilute to the mark with double-distilled water.

c) Dissolve 1,2 g of tetrabutylphosphonium chloride and 0,5 g of sodium dihydrogen phosphate in 850 ml of double-distilled water and add 150 ml of acetonitrile.

Annex D
(informative)

Examples of suitable IC conditions

Table D.1 — Examples of suitable IC conditions

IC Conditions	a	b	c	d
Column	MetrosepASupp 5	Dionex™ IonPac™ AS 14A [a]	MetrosepASupp 5	Dionex™ IonPac™ AS 11-HC with precolumn IonPac AG 11-HC
Column dimension in mm	4 × 50	3 × 150	4 × 250	2 × 250 2 × 50 (precolumn)
Mobile phase	Na_2CO_3 (c = 1,4 mmol/l)/ $NaHCO_3$ (c = 1,1 mmol/l)	Na_2CO_3 (c = 8,0 mmol/l)/ $NaHCO_3$ (c = 1,0 mmol/l)	Na_2CO_3 (c = 3,2 mmol/l)/ $NaHCO_3$ (c = 1,0 mmol/l)	Water (A)/ NaOH (c = 200 mmol/l) (B) see Table D.2
Flow rate in ml/min	0,6 to 0,7	0,5 to 1	0,6 to 0,7	0,35
Injection volume in µl	20	10 to 50	20 to 100	25
Suppressor/ Dialysis	Dialysis/ Suppressor	Suppressor (2 mm)	Dialysis/ Suppressor	Suppressor (2 mm)
Detection	Conductivity detector	Conductivity detector or UV (206 nm)	Conductivity detector or UV (220 nm)	UV (215 nm) DAD

[a] Dionex™ IonPac™ AS 14a or AS11 HC or AG11 HC are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not consitute an endorsement by CEN.

Production of mobile phase:

a) Weigh 2,967 6 g of sodium carbonate (Na_2CO_3) and 1,848 2 g of sodium hydrogen carbonate ($NaHCO_3$) to an accuracy of 1 mg into a 100 ml volumetric flask, dissolve in water and dilute to the mark (0,28 mol/l Na_2CO_3 + 0,22 mol/l $NaHCO_3$). The solution is stable for no more than two months stored at 4 °C. Pipette 10 ml of the above solution into a 2 000 ml volumetric flask, dilute to the mark with water and mix.

b) Weigh 52,995 g of sodium carbonate (Na_2CO_3) and 42,005 g of sodium hydrogen carbonate ($NaHCO_3$) to an accuracy of 1 mg individually into 1 000 ml volumetric flasks, dissolve in water and dilute each flask to the mark (0,5 mol/l Na_2CO_3; 0,5 mol/l $NaHCO_3$). The solutions are stable for no more than two months stored at 4 °C. Pipette 16 ml of the above sodium carbonate solution and 2 ml of the above sodium hydrogen carbonate solution into a 1 000 ml volumetric flask, dilute to the mark with water and mix.

c) Weigh 8,475 g of sodium carbonate (Na_2CO_3) and 2,100 g of sodium hydrogen carbonate ($NaHCO_3$) to an accuracy of 1 mg into a 250 ml volumetric flask, dissolve in water and dilute to the mark (0,32 mol/l Na_2CO_3 + 0,1 mol/l $NaHCO_3$). The solution is stable for no more than two months stored at 4 °C. Pipette 20 ml of the above solution into a 2 000 ml volumetric flask, dilute to the mark with water and mix.

Table D.2 — Composition of the mobile phases (see Table D.1, Column d)

Time min	**Eluent (A)** %	**Eluent (B)** %
0	97	3
> 0 to < 25	90	10
25 to 41	97	3

Bibliography

[1] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

[2] Commission Regulation (EC) No 1882/2006 of 19 December 2006 laying down methods of sampling and analysis for the official control of the levels of nitrates in certain foodstuffs

[3] ISO 5725-3, *Accuracy (trueness and precision) of measurement methods and results — Part 3: Intermediate measures of the precision of a standard measurement method*

[4] EN ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025)*

[5] EN 12014-1, *Foodstuffs — Determination of nitrate and/or nitrite content — Part 1: General considerations*

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