BS EN 14122:2014

BSI Standards Publication

Foodstuffs — Determination of vitamin B1 by high performance liquid chromatography

bsi.

...making excellence a habit.™

National foreword

This British Standard is the UK implementation of EN 14122:2014. It supersedes BS EN 14122:2003 which is withdrawn.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.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Published by BSI Standards Limited 2014

ISBN 978 0 580 77939 8
ICS 67.050

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 June 2014.

Amendments/corrigenda issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14122

June 2014

ICS 67.050

Supersedes EN 14122:2003

English Version

Foodstuffs - Determination of vitamin B1 by high performance liquid chromatography

Produits alimentaires - Détermination de la teneur en vitamine B1 par chromatographie liquide haute performance

Lebensmittel - Bestimmung von Vitamin B1 mit Hochleistungs-Flüssigchromatographie

This European Standard was approved by CEN on 17 April 2014.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

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

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Contents

Page

Foreword

This document (EN 14122:2014) 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 December 2014 and conflicting national standards shall be withdrawn at the latest by December 2014.

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

This document supersedes EN 14122:2003.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

WARNING — The use of this European Standard can involve hazardous materials, operations and equipment. This European Standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this European Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This European Standard specifies a method for the determination of vitamin B_1 in food by high performance liquid chromatography (HPLC) with enzymatic treatment and pre- or post-column derivatization. This method has been validated in two interlaboratory studies. The first study was for the analysis of samples of whole meal flour, milk powder/spray dried milk, freeze-dried mixed vegetables and freeze-dried pig's liver ranging from 0,295 mg/100 g to 0,807 mg/100 g. The second study was for the analysis of samples of tube feeding solution, baby food with vegetables, powdered milk, meal with fruits, yeast, cereal, chocolate powder and food supplement ranging from 0,11 mg/100 g to 486 mg/100 g. Vitamin B_1 is the mass fraction of total thiamin including its phosphorylated derivatives.

For further information on the validation, see Clause 8 and Annex B.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

3 Principle

Thiamin is extracted from food after acid hydrolysis followed by dephosphorylation using an enzymatic treatment and quantified by HPLC with pre- or post-column derivatization to thiochrome. An external standard is used for quantification. For further information see [1] to [7].

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696, or double distilled water.

4.1 Methanol, mass fraction $w(CH_3OH) \geq 99{,}8$ %, HPLC grade.

4.2 Acetic acid solution, substance concentration $c(CH_3COOH) = 0{,}02$ mol/l.

4.3 Isobutanol, $w(C_4H_{10}O) \geq 98$ %.

4.4 Sodium dihydrogen phosphate, $w(NaH_2PO_4) \geq 99{,}8$ %.

4.5 Hydrochloric acid solution, $w(HCl) = 36$ %.

4.6 Hydrochloric acid solution, $c(HCl) = 0{,}1$ mol/l.

4.7 Sulfuric acid solution, $c(H_2SO_4) = 0{,}05$ mol/l.

4.8 Sodium hydroxide, $w(NaOH) \geq 99$ %.

4.9 Sodium hydroxide solution, mass concentration $\rho(NaOH) = 150$ g/l.

4.10 Sodium hydroxide solution, $\rho(NaOH) = 200$ g/l.

4.11 Potassium hexacyanoferrate III, $w\{K_3[Fe(CN)_6]\} \geq 99$ %.

4.12 Potassium hexacyanoferrate III solution, $\rho\{K_3[Fe(CN)_6]\} = 10$ g/l.

4.13 Alkaline potassium hexacyanoferrate III solution (pre-column derivatization), $\rho\{K_3[Fe(CN)_6]\} = 0{,}4$ g/l.

Dilute 2,0 ml of the potassium hexacyanoferrate III solution (4.12) to 50 ml with sodium hydroxide solution (4.9). Prepare fresh each day of analysis.

4.14 Alkaline potassium hexacyanoferrate III solution (post-column derivatization), $\rho\{K_3[Fe(CN)_6]\} = 0{,}5$ g/l.

Dilute 2,5 ml of the potassium hexacyanoferrate III solution (4.12) to 50 ml with sodium hydroxide solution (4.10).

4.15 Enzyme or enzyme mixture, with the ability to liberate vitamin B_1 from foods as free thiamin.

NOTE 1 For the precision data in Table B.1, Taka-Diastase from Pfaltz and Bauer[1)] has been used. For the precision data in Table B.2 and Table B.3 an enzyme mixture of β-amylase from barley and Taka-Diastase from Serva[1)] have been used.

NOTE 2 If incomplete dephosphorylation occurs, this can be solved by the separate quantification of TMP (Thiamin Mono Phosphate), see [7].

4.16 Sodium acetate solution, $c(CH_3COONa \cdot 3H_2O) = 2{,}5$ mol/l.

4.17 Sodium acetate solution, $c(CH_3COONa \cdot 3H_2O) = 0{,}5$ mol/l.

4.18 HPLC mobile phases

Examples of appropriate mixtures with volume fractions of e.g. 10 % to 50 % methanol (4.1) in water or using phosphate or acetate buffer are given in Annex A and Annex C. The possibility of using ion pairing agents is also given.

4.19 Phosphate buffer (pH = 3,5), $c(KH_2PO_4) = 9{,}0$ mmol/l.

4.20 Tetraethylammoniumchloride, $w(C_8H_{20}NCl) \geq 98$ %.

4.21 Sodium heptanesulfonate, $w(C_7H_{15}NaO_3S) \geq 98$ %.

4.22 Acetate buffer (pH = 4,0), $c(CH_3COOH) = 50$ mmol/l.

4.23 Standard substances

4.23.1 Thiamin chloride hydrochloride, $w(C_{12}H_{17}ClN_4OS \cdot HCl) \geq 99$ %.

For external calibration, see 6.3.

4.23.2 Thiamin monophosphate chloride, $w(C_{12}H_{17}ClN_4O_4PS) \geq 98$ %.

For check of enzymes, see 6.2.2.

4.23.3 Thiamin pyrophosphate chloride (cocarboxylase), $w(C_{12}H_{19}ClN_4O_7P_2S) \geq 98$ %.

---

1) The information of the suppliers of Taka-Diastase, Pfaltz & Bauer, Waterbury, CT 06708, USA (No T00040), and Serva is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

For check of enzymes, see 6.2.2.

4.24 Stock solutions

4.24.1 Thiamin chloride hydrochloride stock solution, $\rho(C_{12}H_{17}ClN_4OS \cdot HCl) \approx 0{,}1$ mg/ml.

Dissolve an accurately weighed amount of the thiamin chloride hydrochloride standard substance (4.23.1) in a defined volume of an appropriate solvent, for example 10 mg of vitamin B_1 standard substance in 100 ml of hydrochloric acid solution (4.6). This solution can be stored for four weeks at + 4 °C.

4.24.2 Thiamin monophosphate stock solution, $\rho(C_{12}H_{17}ClN_4O_4PS) \approx 0{,}1$ mg/ml.

Dissolve an accurately weighed amount of the thiamin monophosphate chloride (4.23.2) in a defined volume of an appropriate solvent, for example 10 mg of thiamin monophosphate chloride in 100 ml of hydrochloric acid solution (4.6). This solution can be stored for four weeks at - 20 °C.

4.24.3 Thiamin pyrophosphate stock solution, $\rho(C_{12}H_{19}ClN_4O_7P_2S) \approx 0{,}1$ mg/ml.

Dissolve an accurately weighed amount of the thiamin pyrophosphate chloride (4.23.3) in a defined volume of an appropriate solvent, for example 10 mg of the thiamin pyrophosphate chloride in 100 ml of hydrochloric acid solution (4.6).

4.24.4 Concentration tests - thiamin chloride hydrochloride

Dilute 10 ml of the thiamin chloride hydrochloride solution (4.24.1) with hydrochloric acid solution (4.6) in a 100 ml volumetric flask to the mark. Measure the absorbance of this solution at the maximum of about 247 nm (A_{247}) in a 1 cm cell against hydrochloric solution (4.6) in the reference cell using an UV spectrometer (5.1). Calculate the mass concentration, ρ, in microgram per millilitre thiamin chloride hydrochloride solution (4.24.1) using Formula (1):

$$\rho = \frac{A_{247} \cdot M \cdot 1\,000}{\varepsilon} \qquad (1)$$

where

- ε is the molar absorption coefficient of thiamin chloride hydrochloride at the maximum wavelength of about 247 nm. The value is 14 200 l · mol^{-1} · cm^{-1}. This value is calculated from the extinction coefficient, $E_{1cm}^{1\%}$ = 421, in 0,1 mol/l HCl [8][7] and the molar mass, M = 337,21. The value is given with four significant figures;
- M is the molar mass, in grams per mol. The value is 337,21;
- A_{247} is the absorption value of the thiamin chloride hydrochloride solution.

4.25 Standard solutions

4.25.1 Thiamin chloride hydrochloride standard solution, $\rho(C_{12}H_{17}ClN_4OS \cdot HCl) \approx 1$ µg/ml to 10 µg/ml.

Pipette 1 ml to 10 ml of the thiamin chloride hydrochloride solution (4.24.1) into a 100 ml volumetric flask and dilute to the mark with the appropriate solvent, e.g. hydrochloric acid solution (4.6). This solution can be stored at 4 °C in the dark for 1 month.

4.25.2 Thiamin monophosphate standard solution, $\rho(C_{12}H_{17}ClN_4O_4PS) \approx 1$ µg/ml to 10 µg/ml.

Pipette 1 ml to 10 ml of the thiamin monophosphate solution (4.24.2) into a 100 ml volumetric flask and dilute to the mark with the appropriate solvent, e.g. hydrochloric acid solution (4.6). This solution can be stored at 4 °C in the dark for 1 month.

4.25.3 Thiamin pyrophosphate standard solution, $\rho(C_{12}H_{19}ClN_4O_7P_2S) \approx 1$ µg/ml to 10 µg/ml.

Pipette 1 ml to 10 ml of the thiamin pyrophosphate solution (4.24.3) into a 100 ml volumetric flask and dilute to the mark with the appropriate solvent, e.g. hydrochloric acid solution (4.6). This solution can be stored at 4 °C in the dark for 1 month.

5 Apparatus

Usual laboratory apparatus, glassware, and the following:

5.1 UV spectrometer, UV spectrometer, capable of measuring absorption at defined wavelengths (247 nm), with appropriate cells, e.g. of 1 cm length.

5.2 Autoclave or heating device, autoclave for extraction purpose, e.g. pressure cooker type, with pressure or temperature reading device, electrical heating device or water bath.

5.3 HPLC system

HPLC system, consisting of a pump, a sample injecting device, a fluorescence detector with an excitation and emission wavelength set at e.g. 366 nm and 435 nm, respectively (see Annex C), and an evaluation system such as an integrator.

5.4 HPLC column

5.4.1 General

Other particle sizes or column dimensions than those specified in this European Standard may be used. Separation parameters shall be adapted to such materials to guarantee equivalent results. The performance criterion for suitable analytical columns is the baseline resolution of the thiamin from interferences[2)].

5.4.2 Pre-column oxidation

Analytical columns, e.g. Lichrospher® 60 RP Select B [2)], particle size of 5 µm, diameter 4,0 mm to 4,6 mm, length 100 mm to 250 mm.

5.4.3 Post-column oxidation

Analytical columns, e.g. Supelco® LC-18- DB [2)], particle size of 5 µm, diameter 4,0 mm to 4,6 mm, length 100 mm to 250 mm.

5.5 Filter device

Filtering of the mobile phase as well as of the sample solution through a membrane filter with, e.g. a pore size of 0,45 µm, prior to use or injection will increase longevity of the columns.

5.6 Post-column reactor pump and derivatization tube, a suitable reagent delivery system, a T-type connecting tube and a derivatization tube (e.g. 10 m x 0,33 mm).

[2)] Suitable silica column packing materials available commercially are Lichrosorb® Si 60, Spherisorb® Si, Hypersil® Si and Lichrospher® 100 DIOL. Suitable RP column packing materials are Spherisorb® ODS, µ-Bondapak® radial C18, Supelco® LC-18- DB and Hypersil® ODS. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

6 Procedure

6.1 Preparation of the test sample

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as pre-cooling shall be taken to avoid exposing to high temperature for long periods of time.

6.2 Preparation of the sample test solution

6.2.1 Extraction

Weigh an appropriate amount of the test sample to the nearest mg, e.g. 2 g to 10 g in a conical flask. Add a defined volume ranging from 60 ml to 200 ml of hydrochloric acid solution (4.6), or sulfuric acid solution (4.7). The pH of the solution should not be higher than pH = 2,0. Cover the container with a watch glass and either autoclave the test portion at 121 °C for 30 min, or heat it at 100 °C for 60 min.

The data from the BCR study have shown that a wide range of conditions for the acid hydrolysis can be applied (temperature 95 °C to 130 °C, time 15 min to 60 min). The higher the temperature is, the shorter the time should be.

6.2.2 Enzyme treatment

After cooling to room temperature adjust the extract to the optimal pH for the enzyme used with sodium acetate solution (4.16) or (4.17) and add a suitable amount of enzyme or enzyme mixture (4.15) to the sample. Incubate the mixture at the optimal time and temperature for the enzyme(s) used. After cooling to room temperature transfer the solution to a volumetric flask using distilled water, or another appropriate solvent and dilute the sample test solution to a defined volume (V_{ts}).

For each enzyme used, optimal pH, incubation time and incubation temperature shall be checked.

To ensure an optimal dephosphorylation, the enzymatic step shall be checked with analysis of samples spiked with thiamin monophosphate chloride (4.23.2) or thiamin pyrophosphate chloride (4.23.3), and a material similar in sample type as the test sample. This material should be a certified reference material.

The amount of thiamin possibly brought in with the enzyme(s) (4.15) shall be considered in the calculation of the result.

NOTE For determination of the precision data given in this European Standard in Table B.1, Table B.2 and Table B.3, Taka-Diastase and Taka-Diastase combined with β-amylase from barley was used for dephosphorylation under the following conditions. The extract was adjusted to pH = 4,0 and pH = 4,5, respectively, with sodium acetate solution (4.16) or (4.17) and 100 mg of Taka-Diastase and 10 mg β-amylase per gram of sample was added. The mixture was incubated at 37 °C to 45 °C for 4 h to 24 h, see [5], [10] and [16].

6.2.3 Sample test solution

If necessary, filter the sample solution (6.2.2) through a filter paper or a 0,45 µm membrane filter. Centrifugation may also be used. This is the sample test solution for oxidation (6.3.2 or 6.3.3).

6.3 Oxidation of thiamin to thiochrome

6.3.1 General

The oxidation may be performed either pre-column (6.3.2) or post-column (6.3.3).

6.3.2 Pre-column oxidation

6.3.2.1 Procedure for oxidation step

Pipette 1 ml of the enzymatically treated sample (6.2.3), standard (4.25.1) or blank i.e. hydrochloric acid solution (4.6) or sulfuric acid solution (4.7) depending which was used in 6.2.1 into suitable vials or flasks, add 1 ml of alkaline hexacyanoferrate III solution (4.13). Shake the sample test solution for a fixed amount of time (e.g. 10 s), leave to stand for a specified time (e.g. 1 min).

In order to remove interfering compounds and to protect the HPLC column it is recommended to neutralize the sample test solution (e.g. with H_3PO_4) or to perform a clean-up using solid phase extraction (for more information see [5]).

Filter through a 0,45 µm membrane filter. This is the sample test solution to inject into the reverse phase HPLC system (6.3.2.2).

Alternatively, the oxidized solution can be extracted into 1,5 ml isobutanol (4.3) and the extract can be injected.

NOTE The oxidative conversion of thiamin to thiochrome can be inhibited in some foods. This phenomena is often encountered in cocoa containing foods, but can also be observed in other foods. If such a problem is suspected, it is recommended to check the recovery of the method by spiking the sample extract with an appropriate volume of thiamin standard solution before the oxidation reaction.

6.3.2.2 Identification with HPLC after pre-column oxidation

Inject the same appropriate volumes of the solutions (6.3.2.1) of standards, samples and blank into the HPLC system. Identify the thiochrome by comparison of the retention time of the individual peaks in the chromatograms obtained with the sample test solution, and with the standard test solution. Adding the standard substances to the sample test solution can also perform peak identification.

The separation and the quantification were proven to be satisfactory if the following experimental conditions are followed (see Annex C and Figure A.1 for alternative HPLC conditions and examples of chromatograms).

Stationary phase:	Lichrospher® RP Select B, 5 µm, 250 mm x 4,0 mm
Mobile phase:	40 parts per volume of methanol (4.1) and 60 parts per volume of acetate buffer (4.22)
Flow rate:	0,7 ml/min
Injection volume:	20 µl
Detection:	Fluorometric: excitation: 366 nm and emission: 435 nm

6.3.3 Post-column oxidation

6.3.3.1 Procedure for oxidation step

Oxidize thiamin to thiochrome using a post-column reaction with the alkaline potassium hexacyanoferrate III solution (4.14). Add continuously (e.g. 0,3 ml/min) the derivatization reagent through a T-type connecting tube to the HPLC eluent to form the thiochrome.

NOTE The post-column oxidation step is influenced e.g. by the sodium hydroxide concentration. Higher concentrations in the derivatization solution can be compensated by a lower pump rate and vice versa.

6.3.3.2 Identification with HPLC using post-column oxidation

Inject the same appropriate volumes of the standards of thiamin chloride hydrochloride (4.23.1) as well as of the sample solutions (6.2.3) into the HPLC system. Identify the thiamin by comparison of the retention time of the

individual peaks in the chromatograms obtained with the sample test solution, and with the standard (4.23.1). Adding thiamin chloride hydrochloride to the sample test solution can also perform peak identification.

The separation and the quantification were proven to be satisfactory if the following experimental conditions are followed (see Annex C and Figure A.2 for alternative HPLC conditions and examples of chromatograms).

Stationary phase:	Supelco® LC-18-DB, 5 µm, 250 mm x 4,6 mm
Mobile phase:	Methanol (4.1): phosphate buffer (4.19), containing 1 g/l tetraethylammoniumchloride (4.20) and 5 mmol/l sodium heptanesulfonate (4.21) (35:65)
Flow rate:	1,0 ml/min
Injection volume:	20 µl
Post-column reagent	Alkaline potassium hexacyanoferrate III solution (4.14)
Reagent flow:	0,3 ml/min
Detection:	Fluorometric: excitation: 368 nm; emission: 440 nm

NOTE Analysis of some samples e.g. raw pork can give an additional peak of 2(1-hydroxyethyl) thiamin in the chromatogram see Annex D.

6.4 Determination

Carry out a determination by external calibration using either integration of the peak areas (preferable) or determine the peak heights (optional) of the sample and compare the results with the corresponding values for the thiochrome by use of a calibration graph. Check the linearity of the calibration.

7 Calculation

Base the calculation on a calibration graph or use the corresponding programmes of the integrator or use the following simplified procedure. Calculate the mass fraction, *w*, of vitamin B_1 expressed as thiamin chloride hydrochloride in mg/100 g of the sample using Formula (2):

$$w = \frac{A_{ts} \cdot \rho \cdot V_{ts}}{A_{st} \cdot m_s} \cdot \frac{100}{1\,000} \qquad (2)$$

where

A_{ts} is the peak area or peak height for thiochrome obtained with the sample test solution, in units of area or height;

A_{st} is the peak area or peak height thiochrome obtained with the standard test solution, in units of area or height;

V_{ts} is the volume of sample test solution (6.2.2), in millilitre;

ρ is the mass concentration of thiamin chloride hydrochloride in the standard solution (4.25.1), in microgram per millilitre;

m_s is the sample mass, in gram (6.2.1);

100 is the factor to calculate the content per 100 g;

1 000 is the factor to convert µg/100 g to mg/100 g.

Report the result for vitamin B_1 in mg/100 g expressed as thiamin chloride hydrochloride (M = 337,28). If it is necessary to express the result as thiamin ($C_{12}H_{17}N_4OS$, M = 265,37) multiply the result with the factor 0,787, as thiamin chloride ($C_{12}H_{17}ClN_4OS$, M = 300,82) multiply with the factor 0,892.

8 Precision

8.1 General

The precision data for the method is partly based on different HPLC methods applied for the determination of thiamin in an international comparison study organized on behalf of the European Commission's Standards Measurement and Testing Programme on a sample of whole meal flour (CRM 121), milk powder/spray dried milk (CRM 421), freeze-dried mixed vegetables (CRM 485) and freeze-dried lyophilised pig liver (CRM 487). The study provided the statistical information shown in Annex B, Table B.1. Furthermore, the precision data include the results of a French collaborative study in tube feeding solution, baby food with vegetables, powdered milk, meal with fruits, yeast, cereal, chocolate powder and food supplement. The results from this study are shown in Annex B, Table B.2 and Table B.3.

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 for thiamin chloride hydrochloride are:

Whole meal flour	$\bar{x}$ = 0,452 mg/100 g	r = 0,043 mg/100 g
Milk powder/spray dried milk	$\bar{x}$ = 0,645 mg/100 g	r = 0,071 mg/100 g
Freeze-dried mixed vegetables	$\bar{x}$ = 0,295 mg/100 g	r = 0,039 mg/100 g
Freeze-dried pig's liver	$\bar{x}$ = 0,807 mg/100 g	r = 0,088 mg/100 g
Tube feeding solution	$\bar{x}$ = 0,11 mg/100 g	r = 0,02 mg/100 g
Baby food with vegetables	$\bar{x}$ = 0,20 mg/100 g	r = 0,05 mg/100 g
Powdered milk	$\bar{x}$ = 0,56 mg/100 g	r = 0,1 mg/100 g
Meal with fruits	$\bar{x}$ = 1,04 mg/100 g	r = 0,2 mg/100 g
Yeast	$\bar{x}$ = 1,31 mg/100 g	r = 0,34 mg/100 g
Cereal	$\bar{x}$ = 1,42 mg/100 g	r = 0,16 mg/100 g
Cereal	$\bar{x}$ = 2,95 mg/100 g	r = 0,49 mg/100 g
Chocolate powder	$\bar{x}$ = 1,55 mg/100 g	r = 0,36 mg/100 g
Food supplement	$\bar{x}$ = 486 mg/100 g	r = 111 mg/100 g

8.3 Reproducibility

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

The values for thiamin chloride hydrochloride are:

Whole meal flour	$\bar{x}$ = 0,452 mg/100 g	*R* = 0,190 mg/100 g
Milk powder/spray dried milk	$\bar{x}$ = 0,645 mg/100 g	*R* = 0,243 mg/100 g
Freeze-dried mixed vegetables	$\bar{x}$ = 0,295 mg/100 g	*R* = 0,178 mg/100 g
Freeze-dried pig's liver	$\bar{x}$ = 0,807 mg/100 g	*R* = 0,623 mg/100 g
Tube feeding solution	$\bar{x}$ = 0,11 mg/100 g	*R* = 0,1 mg/100 g
Baby food with vegetables	$\bar{x}$ = 0,20 mg/100 g	*R* = 0,12 mg/100 g
Powdered milk	$\bar{x}$ = 0,56 mg/100 g	*R* = 0,25 mg/100 g
Meal with fruits	$\bar{x}$ = 1,04 mg/100 g	*R* = 0,55 mg/100 g
Yeast	$\bar{x}$ = 1,31 mg/100 g	*R* = 0,48 mg/100 g
Cereal	$\bar{x}$ = 1,42 mg/100 g	*R* = 0,75 mg/100 g
Cereal	$\bar{x}$ = 2,95 mg/100 g	*R* = 1,16 mg/100 g
Chocolate powder	$\bar{x}$ = 1,55 mg/100 g	*R* = 0,8 mg/100 g
Food supplement	$\bar{x}$ = 486 mg/100 g	*R* = 212 mg/100 g

9 Test report

The test report should comply with EN ISO/IEC 17025 [17] and shall contain at least the following data:

a) all information necessary for the identification of the sample;

b) a reference to this European Standard or to the method used;

c) the date and time of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

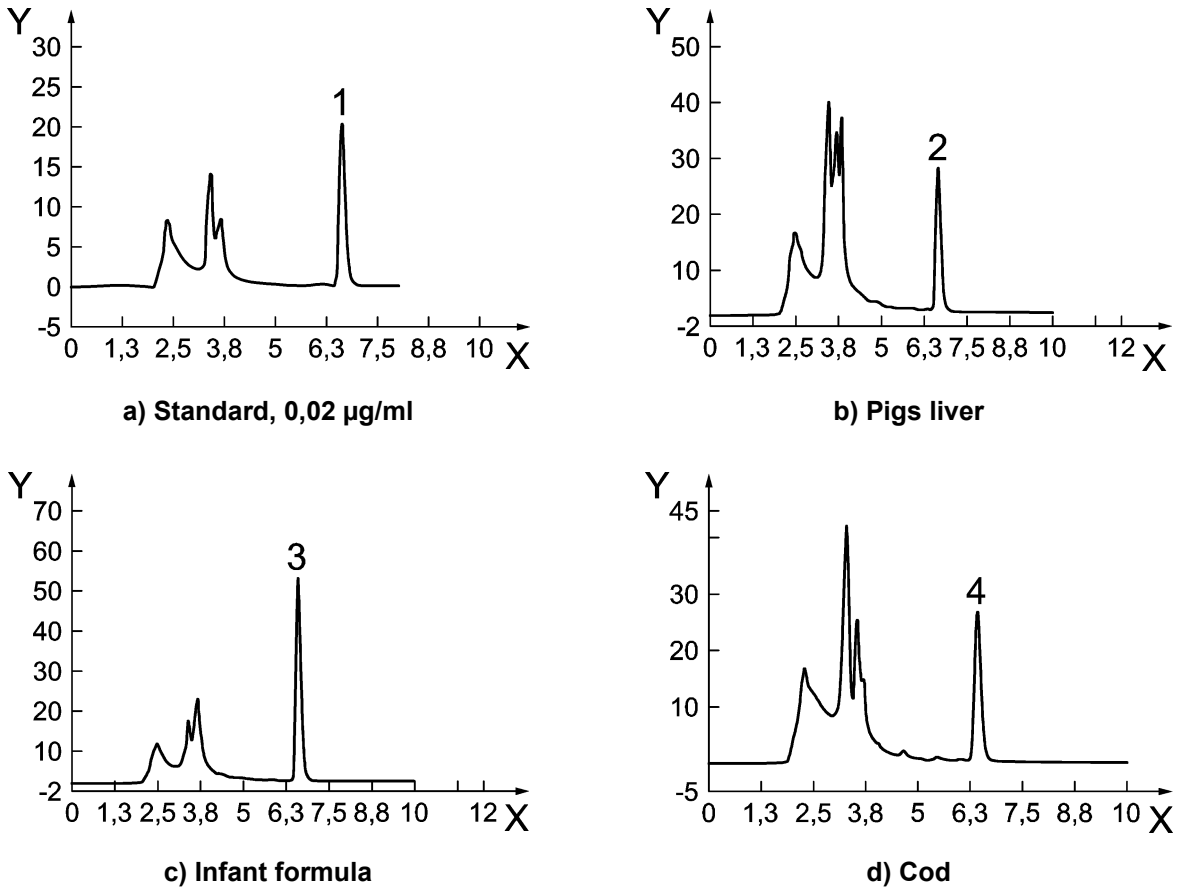
f) the results and the units in which the results have been expressed;

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 chromatograms



a) Standard, 0,02 µg/ml

b) Pigs liver

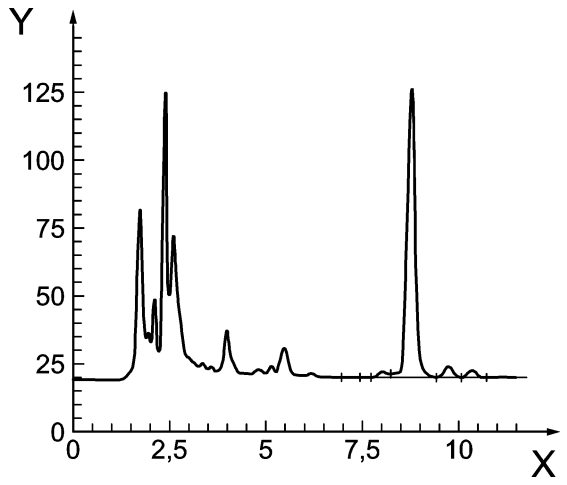
c) Infant formula

d) Cod

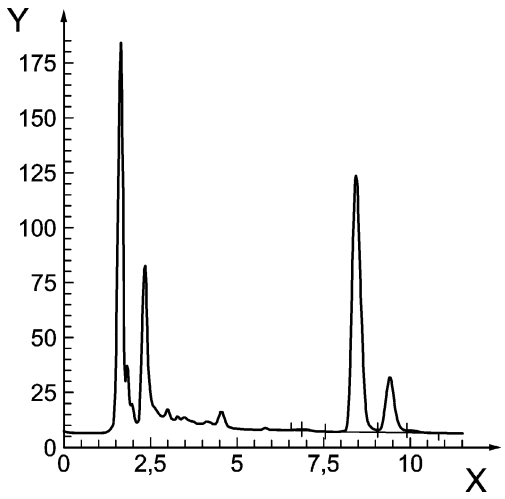
Key

Y	fluorescence
X	time (min)
1	thiamin in standard, 0,02 µg/ml at 6,620 min
2	thiamin in pigs liver at 6,616 min
3	thiamin in infant formula at 6,619 min
4	thiamin in cod at 6,614 min
Stationary phase:	Gemini C18, 5 µm, 250 mm x 4,6 mm (110 Å)
Mobile phase:	methanol (4.1): water pH 9 (40:60)
Flow rate:	0,8 ml/min
Injection volume:	20 µl
Detection:	fluorometric: excitation: 366 nm; emission: 435 nm

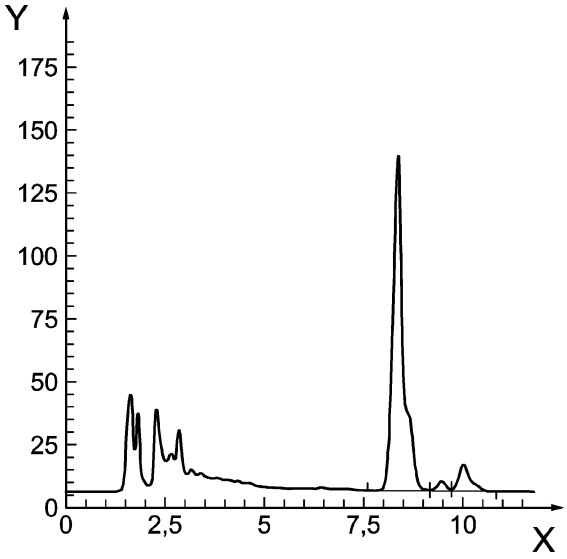
Figure A.1 — Example for an HPLC separation of thiamin as thiochrome standard using pre-column derivatization



a) Lettuce



b) Cooked rice



c) Cooked pork meat

Key

Y	fluorescence
X	time (min)

Stationary phase:	Purospher® RP C18, endcapped, 5 µm, 250 mm x 4,6 mm
Mobile phase:	Methanol (4.1): phosphate buffer, pH 3,5, $c(NH_4H_2PO_4)$ = 10 mmol/l, containing 1 g/l tetraethylammoniumchloride (4.20), and 5 mmol/l sodium heptanesulfonate (4.21), (35:70)
Flow rate:	1,5 ml/min
Injection volume:	3 µl
Post-column reagent:	Alkaline potassium hexacyanoferrate III solution (4.14)
Reagent flow:	0,3 ml/min
Detection:	fluorometric: excitation: 365 nm; emission: 435 nm

NOTE Additional chromatogram for meat/liver is given in Annex D.

Figure A.2 — Examples for a HPLC separation of thiamin using post-column derivatization in lettuce (a), cooked rice (b) and cooked pork meat (c)

Annex B
(informative)

Precision data

In accordance with the EU SMT Certification Study Guidelines, the data given in Table B.1 have been defined in an interlaboratory test [10]. The Institute of Food Research, Norwich, UK on behalf of the EU Community Bureau of Reference, conducted the study. The data given in Table B.2 and Table B.3 have been defined in a French interlaboratory test [5].

Table B.1 — Precision data for whole meal flour, milk powder/spray dried milk, freeze-dried mixed vegetables and freeze-dried pig's liver

Samples	**CRM 121** **Whole meal flour**	**CRM 421** **Milk powder/ spray dried fresh milk**	**CRM 485** **Freeze-dried mixed vegetables**[a]	**CRM 487** **Freeze-dried pig's liver**
Year of interlaboratory study	1996	1996	1996	1996
Number of laboratories	13	14	12	15
Number of samples	2	2	2	2
Number of laboratories retained after eliminating outliers	13	14	12	15
Number of outliers	0	0	0	0
Number of accepted results	65	70	58	72
Mean value, $\bar{x}$, mg/100 g	0,452	0,645	0,295	0,807
Repeatability standard deviation, s_r, mg/100 g	0,015	0,025	0,012	0,031
Repeatability coefficient of variation, %	3,2	3,8	4,2	3,9
Repeatability value, r [$r = 2{,}83 \times s_r$], mg/100 g	0,043	0,071	0,039	0,088
Reproducibility standard deviation, s_R, mg/100 g	0,053	0,085	0,063	0,182
Reproducibility coefficient of variation, %	11,8	13,2	13,3	22,6
Reproducibility value R, [$R = 2{,}83 \times s_R$], mg/100 g	0,190	0,243	0,178	0,623
HorRat value, according to [13]	0,9	1,1	1,0	1,9
[a] Mixture of sweetcorn, carrots and drained tomatoes (10:1:1)				

NOTE The data obtained in this international comparison study have been produced using established methods being identical with in-house routine assay procedures of the participating laboratories with the HPLC systems described in Annex C.

Table B.2 — Precision data for tube feeding solution, baby food with vegetables, powdered milk, meal with fruits and yeast

Samples	**Tube feeding solution**	**Baby food with vegetables**	**Powdered Milk**	**Meal with fruits**	**Yeast**
Year of study	1995	1995	1995	1995	1995
Number of laboratories	10	10	10	10	10
Number of samples	1	1	1	1	1
Number of laboratories retained after eliminating outliers	8	10	10	10	10
Number of outliers	2	0	0	0	0
Number of accepted results	16	20	20	20	20
Mean value, $\bar{x}$, mg/100 g	0,11	0,20	0,56	1,04	1,31
Repeatability standard deviation s_r	0,01	0,02	0,04	0,07	0,12
Repeatability coefficient of variation	7 %	8 %	7 %	7 %	9 %
Repeatability value r [$r = 2{,}83 \times s_r$], mg/100 g	0,02	0,05	0,1	0,2	0,34
Reproducibility standard deviation s_R, mg/100 g	0,04	0,04	0,08	0,19	0,17
Reproducibility coefficient of variation	32 %	21 %	16 %	19 %	13 %
Reproducibility value R [$R = 2{,}83 \times s_R$], mg/100 g	0,1	0,12	0,25	0,55	0,48
HorRat value, according to [13]	2,0	1,5	1,3	1,7	1,2

Table B.3 — Precision data for cereal, chocolate powder and food supplement

Samples	**Cereal**	**Cereal**	**Chocolate powder**	**Food supplement**
Year of study	1995	1995	1995	1995
Number of laboratories	10	10	10	10
Number of samples	1	1	1	1
Number of laboratories retained after eliminating outliers	9	9	9	9
Number of outliers	1	1	1	1
Number of accepted results	18	18	18	18
Mean value, $\bar{x}$, mg/100 g	1,42	2,95	1,55	486
Repeatability standard deviation s_r	0,06	0,18	0,13	39
Repeatability coefficient of variation	4 %	6 %	8 %	8 %
Repeatability value r [$r = 2{,}83 \times s_r$], mg/100 g	0,16	0,49	0,36	111
Reproducibility standard deviation s_R, mg/100g	0,27	0,41	0,28	75
Reproducibility coefficient of variation	19 %	14 %	19 %	15 %
Reproducibility value R [$R = 2{,}83 \times s_R$], mg/100 g	0,75	1,16	0,8	212
HorRat value, according to [13]	1,8	1,5	1,8	3,4[a]

[a] In 1980, Horwitz et al. published an evaluation of 1 000 interlaboratory comparisons. From these studies, it was concluded that a HorRat value of 1, with limits of acceptability between 0,5 and 2,0, indicates satisfactory interlaboratory precision. The corresponding within laboratory relative standard deviations were found to be typically one-half to two-thirds the among-laboratory relative standard deviations. Consistent deviations from the ratio on the low side (values < 0,3 or 0,5) may indicate unreported averaging or excellent training and experience.

Annex C
(informative)

Alternative HPLC systems

The separation and quantification has proven to be satisfactory if the following chromatographic conditions are being applied [10].

Table C.1 — Alternative HPLC conditions

Stationary phase	**Column dimensions** mm x mm	**Mobile phase** (*V*:*V*)	**Detection** (Ex/Em) in nm	**Flow** ml/min	**Oxidation mode**
Radial silica® 10 µm	250 × 4,6	Ethanol: phosphate buffer pH 7,4, $c(K_2HPO_4)$ = 0,1 mol/l (50:50)	F: 365/435	3,0	PC[a]
Supelco® LC-18-DB 5 µm	250 × 4,6	Methanol: phosphate buffer; pH 3,5, $c(KH_2PO_4)$ = 9 mmol/l containing tetraethylammoniumchloride, $\rho(C_8H_{20}NCl)$ = 1 g/l and sodium heptanesulfonate, $c(C_7H_{15}NaO_3S)$ = 5 mmol/l (35:65)	F: 368/420	1,0	PC
Lichrospher® RP18 5 µm	250 × 4,6	Methanol: sodium hexanesulfonate, $c(C_6H_{13}NaO_3S \cdot H_2O)$ = 1 mmol/l, pH 3,0 (70:30)	F: 375/435	1,5	PC
Eurospher® 100-C18 5 µm	250 × 4,6	Sodium dihydrogen phosphate, $c(NaH_2PO_4)$ = 10 mmol/l): sodium perchlorate, $c(NaClO_4)$ = 0,15 mol/l (50:50)	F: 375/435	1,0	PC
Lichrospher® RP Select B 5 µm	250 × 4,6	Methanol: acetate buffer pH 4,0, $c(CH_3COONa)$ = 50 mmol/l (40:60)	F: 366/435	0,7	PRC[b]
µ-Bondapak® radial C18 5 µm	250 × 4,6	Methanol: acetate buffer pH 4,5, $c(CH_3COONa)$ = 0,5 mol/l (40:60)	F: 366/435	0,8	PRC
Spherisorb® ODS2 5 µm	250 × 4,6	Methanol: phosphate buffer pH 4,0, $c(KH_2PO_4)$ = 0,1 mol/l (70:30)	F: 375/435	1,0	PRC
Lichrospher® RP18 10 µm	250 × 4,6	Potassium dihydrogenphosphate, $c(KH_2PO_4)$ = 10 mmol/l / dimethylformamide (80:20)	F: 368:440	1,5	PRC
Hamilton® PRP-1 5 µm	150 × 4,6	Methanol: water (40:60); pH adjusted to 4,5 with acetic acid	F: 366/435	1,0	PRC
Hamilton® PRP-1 5 µm	150 × 4,1	Methanol: water (35:65) pH 9,0; adjusted with ammoniac, $w(NH_3)$ = 25 %	F: 366/435	1,0	PRC
Hypersil® NH_2APS2 5 µm	250 × 4,6	Dichloromethane: methanol (95:5)	F: 365/440	1,0	PRC

[a] PC = Post-column derivatization.

[b] PRC = Pre-column derivatization.

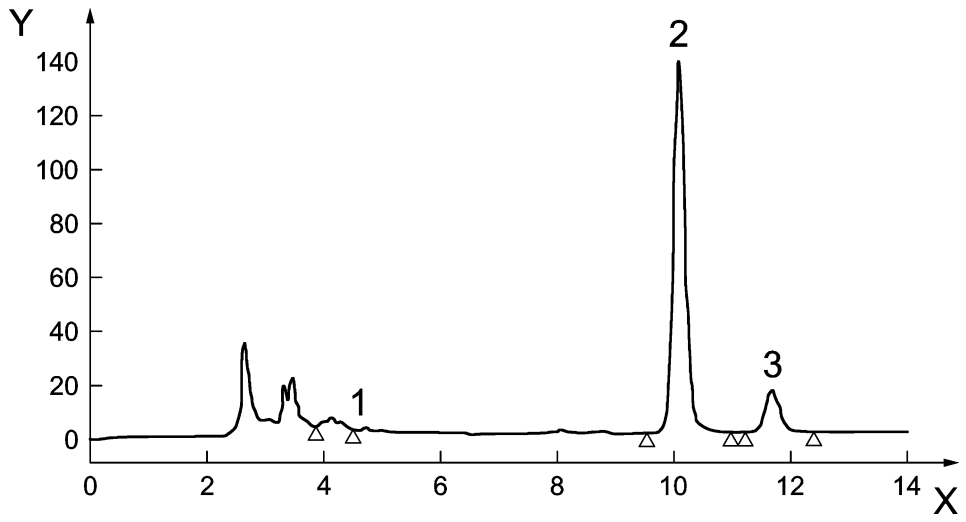
Annex D
(informative)

Vitamin B_1 compound: 2-(1-hydroxyethyl)thiamin (HET) performing post-column derivatization

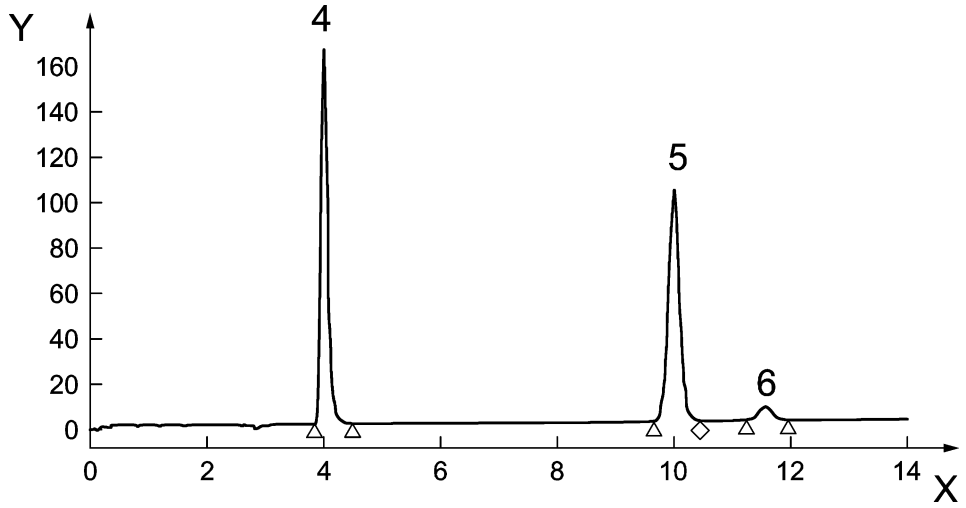
Usually, the vitamin B_1 content has been regarded as thiamin and its phosphate derivatives. However, the use of the post-column derivatization method can show two peaks for thiamin, and the confirmation of the extra metabolite 2-(1-hydroxyethyl)thiamin (HET) was evident, see [11] and [12] and see Figure D.1. If the pre-column derivatization technique is used, the two compounds thiamin and HET co-elute.

The relative content of HET compared to thiamin is dependent on the sample type. In meat and liver the content of HET is 7 % to 23 % of the content of thiamin. In yeast the content was 3,8 %, while in white cabbage, broccoli, oat flour, infant formula, milk powder and wheat the content was neglible below 2 %, see [7].

Quantitative determination of vitamin B_1 in food by a post-column derivatization is recommended to include separate quantitative determination of thiamin and HET when analysing meat samples, and to test other type of samples for the appearance of HET in the chromatogram.



a) Pigs liver



b) Standard

Key

Y fluorescence

X time (min)

1 Thiamin Mono Phosphate (TMP) in pigs liver at 4,034 min

2 Thiamin in pigs liver at 10,136 min

3 2-(1-Hydroxy Ethyl) Thiamin (HET) in pigs liver at 11,741 min

4 TMP in standard at 4,034 min

5 Thiamin in standard at 10,044 min

6 HET in standard at 11,613 min

NOTE HPLC information is given in [7].

Figure D.1 — Chromatogram of an extract of CRM 487 pigs liver (a) and a standard of 0,1 µg/ml TMP (Thiamin Mono Phosphate), 0,1 µg/ml thiamin and 0,01 µg/ml HET (2-(1-hydroxyethyl)thiamin) (b)

Bibliography

[1] BOGNÁR A. Bestimmung von Riboflavin und Thiamine in Lebensmitteln mit Hilfe der Hochleistungsflüssigkeitschromatographie (HPLC). *Dtsch. Lebensmitt. Rundsch.* 1981, **77** pp. 431–436

[2] HASSELMANN C., FRANCK D., GRIMM P., DIOP P.A., SOULES C. High-performance liquid chromatographic analysis of thiamine and riboflavin in dietetic foods. *J. Micronutr. Anal.* 1989, **5** pp. 269–279

[3] BOGNÁR A. Determination of vitamin B1 in food by High-Performance-Liquid-Chromatography and post-column derivatization. *Fresenius J. Anal. Chem.* 1992, **343** pp. 155–156

[4] HÄGG M., KUMPULAINEN J. Thiamine and riboflavin contents in domestic and imported cereal products in Finland. *J. Food Compos. Anal.* 1993, **6** pp. 299–306

[5] ARELLA F., LAHÉLY S., BOURGUIGNON J.B., HASSELMANN C. Liquid chromatographic determination of vitamin B1 and B2 in foods. A collaborative study. *Food Chem.* 1996, **56** pp. 81–86

[6] EITENMILLER R.R., LANDEN W.O. *Vitamin Analysis for the Health and Food Sciences*. CRC Press, Boca Raton, London, New York, Washington, D.C., 1999, pp. 271–97.

[7] JAKOBSEN J. Optimisation of the determination of thiamin, 2-(1-hydroxyethyl)thiamine, and riboflavin in food samples by use of HPLC. *Food Chem.* 2008, **106** pp. 1209–1217

[8] DAWSON R.M.C., ELLIOTT D.C., ELLIOT W.H., JONES K. Data for Biochemical Research. Oxford Science Publication 3rd. ISBN 0 19 855299 8, 1989

[9] HÄGG M. Effect of various commercially available enzymes in the liquid chromatographic determination with external standardization of thiamine and riboflavin in foods. *J. AOAC Int.* 1994, **77** pp. 681–686

[10] FINGLAS P.M., SCOTT K.J., WITTHOFT C.M., VAN DEN BERG H., DE FROIDMONT-GORTZ I. *The certification of the mass fractions of vitamins in four reference materials: Wholemeal flour (CRM 121), milk powder (CRM 421), lyophilised mixed vegetables (CRM 485) and lyophilised pig's liver (CRM 487). EUR-report 18320*. Office for Official Publications of the European Communities, Luxembourg, 1999

[11] TAKASHI U., YUKIKO T., KOHEI M., MARI T., KANAME K. Simultaneous determination of 2(1-hydroxyethyl)thiamine and thiamine in foods by high performance liquid chromatography with post-column derivatisation. *Vitamins (Japan)*. 1990, **64** pp. 379–385

[12] TAKASHI U., YUKIKO T., KOHEI M., MASAKO M., KANAME K. Distribution and stability of 2(1-hydroxyethyl)thiamine and thiamine in foods. *Vitamins (Japan)*. 1991, **65** pp. 249–256

[13] HORWITZ W., ALBERT R. The Horwitz Ratio (HorRat): A useful Index of Method Performance with Respect to Precision. *J. AOAC Int.* 2006, **89** pp. 1095–1109

[14] THOMPSON M. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. *Analyst (Lond.)*. 2000, **125** pp. 385–386

[15] HORWITZ W., KAMPS L.R., BOYER K.W. Quality assurance in the analysis of foods and trace constituents. *J. AOAC Int.* 1980, **63** pp. 1344–1354

[16] OLLILAINEN V., FINGLAS P.M., VAN DEN BERG H., DE FROIDMONT-GÖRTZ I. Certification of B-Group Vitamins (B1, B2, B6, and B12) in Four Food Reference Materials. *J. Agric. Food Chem.* 2001, **49** pp. 315–321

[17] EN ISO/IEC 17025:2005, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005)*

This page deliberately left blank

British Standards Institution (BSI)

BSI is the national body responsible for preparing British Standards and other standards-related publications, information and services.

BSI is incorporated by Royal Charter. British Standards and other standardization products are published by BSI Standards Limited.

About us

We bring together business, industry, government, consumers, innovators and others to shape their combined experience and expertise into standards -based solutions.

The knowledge embodied in our standards has been carefully assembled in a dependable format and refined through our open consultation process. Organizations of all sizes and across all sectors choose standards to help them achieve their goals.

Information on standards

We can provide you with the knowledge that your organization needs to succeed. Find out more about British Standards by visiting our website at bsigroup.com/standards or contacting our Customer Services team or Knowledge Centre.

Buying standards

You can buy and download PDF versions of BSI publications, including British and adopted European and international standards, through our website at bsigroup.com/shop, where hard copies can also be purchased.

If you need international and foreign standards from other Standards Development Organizations, hard copies can be ordered from our Customer Services team.

Subscriptions

Our range of subscription services are designed to make using standards easier for you. For further information on our subscription products go to bsigroup.com/subscriptions.

With **British Standards Online (BSOL)** you'll have instant access to over 55,000 British and adopted European and international standards from your desktop. It's available 24/7 and is refreshed daily so you'll always be up to date.

You can keep in touch with standards developments and receive substantial discounts on the purchase price of standards, both in single copy and subscription format, by becoming a **BSI Subscribing Member**.

PLUS is an updating service exclusive to BSI Subscribing Members. You will automatically receive the latest hard copy of your standards when they're revised or replaced.

To find out more about becoming a BSI Subscribing Member and the benefits of membership, please visit bsigroup.com/shop.

With a **Multi-User Network Licence (MUNL)** you are able to host standards publications on your intranet. Licences can cover as few or as many users as you wish. With updates supplied as soon as they're available, you can be sure your documentation is current. For further information, email bsmusales@bsigroup.com.

BSI Group Headquarters

389 Chiswick High Road London W4 4AL UK

Revisions

Our British Standards and other publications are updated by amendment or revision.

We continually improve the quality of our products and services to benefit your business. If you find an inaccuracy or ambiguity within a British Standard or other BSI publication please inform the Knowledge Centre.

Copyright

Useful Contacts:

Customer Services
Tel: +44 845 086 9001
Email (orders): orders@bsigroup.com
Email (enquiries): cservices@bsigroup.com

Subscriptions
Tel: +44 845 086 9001
Email: subscriptions@bsigroup.com

Knowledge Centre
Tel: +44 20 8996 7004
Email: knowledgecentre@bsigroup.com

Copyright & Licensing
Tel: +44 20 8996 7070
Email: copyright@bsigroup.com

bsi.

...making excellence a habit.™