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**Determination of hydroxytyrosol  
and tyrosol content in extra virgin  
olive oils — Reverse phase high  
performance liquid chromatography  
(RP-HPLC) method**

*Détermination de la teneur en hydroxytyrosol et tyrosol dans les  
huiles d'olive vierges extra — Méthode par chromatographie liquide à  
haute performance en phase inverse (CLHP-RP)*

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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This first edition cancels and replaces ISO/TS 23942:2020, which has been technically revised.

The main changes are as follows:

- conversion of a Technical Specification to an International Standard;
- additional validation studies have been added to [Annex C](#).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

## Introduction

Biophenolic compounds of a secoiridoid nature, and typical of extra virgin olive oil (*Olea europaea* L.), are derived from oleuropein and ligstroside, and are correlated to different beneficial health effects for human beings other than particular sensorial characteristics. The biophenolic compounds contain, in an esterified form, two aromatic alcohols, namely hydroxytyrosol and tyrosol. The method given in this document is based on an extraction of the biophenolic fraction with a methanol/water solution and a subsequent hydrolysis reaction to produce free tyrosol and hydroxytyrosol.<sup>[1][2]</sup>

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# Determination of hydroxytyrosol and tyrosol content in extra virgin olive oils — Reverse phase high performance liquid chromatography (RP-HPLC) method

## 1 Scope

This document specifies a method for the quantitative determination of hydroxytyrosol and tyrosol content in extra virgin olive oils using reverse phase high performance liquid chromatography (RP-HPLC) with spectrophotometric detection.

The method is also applicable to all other olive oils of a different commercial category.

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

### 3.1

#### hydroxytyrosol and tyrosol

aromatic alcohols present in extra virgin olive oil typical of *Olea europaea* L. species as free or bound form

## 4 Principle

Hydroxytyrosol and tyrosol, present in free and esterified forms, are extracted from the oil with a methanol/water solution and then submitted to hydrolysis reaction with a 10 % of sulphuric acid ethanolic solution. The components are identified by means of HPLC and a spectrophotometric detector at 280 nm. The amount of free aromatic alcohols is calculated with the use of an external standard.

## 5 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade.

**5.1 Ortho-phosphoric acid**, a volume fraction of 85 %.

**5.2 Methanol chromatographic grade.**

**5.3 Acetonitrile chromatographic grade.**

**5.4 Water chromatographic grade.**

5.5 **Ethanol**, a volume fraction of 96 %.

5.6 **Sulphuric acid**, a volume fraction of 96 %.

5.7 **Methanol/water solution**, 80/20 v/v.

5.8 **Reference sample: hydroxytyrosol or 2-(3,4-Dihydroxyphenyl)ethanol**, e.g. Extrasynthese, (Cedex, France)<sup>1)</sup>.

5.9 **Reference sample: tyrosol**, e.g. Sigma Aldrich (Germany)<sup>1)</sup>.

5.10 **Standard calibration solution of hydroxytyrosol and tyrosol.**

Prepare the external standard calibration solution of hydroxytyrosol and tyrosol as follows.

Weigh accurately, to the nearest 0,1 mg, about 25 mg of hydroxytyrosol (5.8) and tyrosol (5.9) in a graduated 50 ml flask (6.2) and make to volume with a solution of methanol/water 80/20 v/v (5.7). Transfer 1 ml of this solution in another 10 ml flask and fill to volume with the same solution of methanol/water 80/20 v/v (5.7). The final concentration is 50 mg/l of each external standard. Inject 20 µl of this solution in the HPLC system. The solution is stable for at least six months at -20 °C.

5.11 **Hydrolysis solution**, consisting of ethanol/water/sulphuric acid 50/40/10 v/v/v.

## 6 Apparatus

The usual laboratory glassware and the following shall be used.

6.1 **Analytical balance** suitable for weighing to an accuracy of within ± 0,1 mg.

6.2 **10 ml and 50 ml calibrated flasks**, class A.

6.3 **1 000 µl and 5 000 µl electronic pipette or manual pipette.**

6.4 **10 ml test tube**, with a screw cap.

6.5 **Mixer**, type vortex.

6.6 **Ultrasonic extraction bath.**

6.7 **PVDF (polyvinyl difluoride) syringe filters**, 0,45 µm, 13 mm.

6.8 **Centrifuge**, able to operate at 5 000 r/min.

6.9 **5 ml plastic syringe.**

6.10 **Thermostatic bath.**

6.11 **Analytical system**, comprising a HPLC ternary pump with a degassing system equipped with an HPLC column, RP 18 reverse phase. The following column has proven to be adapted for the

1) Extrasynthese (Cedex, France) and Sigma Aldrich (Germany) are examples of a companies that make suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

determination (internal diameter 4,6 mm, length 25 cm, size 5 µm, 100 Å, type Spherisorb ODS2<sup>2)</sup>) with a UV spectrophotometric detector at 280 nm and integration system. A photodiode array detector (PDA) for spectra recording can be used to facilitate peak identification, by matching the hydroxytyrosol and tyrosol spectra in the sample extract with the spectra of the external standard.

A system for analysis data and integration is needed.

## 7 Sampling

It is important that an intact oil sample is delivered to the laboratory, which has not been damaged or modified during transport or storage. A representative sample is considered for the purpose of the analysis. A recommended sampling method is given in ISO 5555<sup>[3]</sup>.

## 8 Procedure

### 8.1 Sample preparation

Weigh, with an analytical balance (6.1), 2 g of oil well-homogenized in a 10 ml conical test tube (6.4). Add, using a pipette (6.3), 5 ml of the methanol/water solution 80/20 v/v (5.7). Mix the solution with the help of a mixer for test tube type vortex (6.5) for 1 min and continue the extraction for 15 min in an ultrasonic bath (6.6) at room temperature. Centrifuge (6.8) at 5 000 r/min for 25 min. Filter an aliquot through a PVDF membrane syringe filter (6.7). Transfer 1 ml, using a pipette (6.3), of the filtered solution into another 10 ml test tube (6.4) and completely dry on a thermostatic bath (6.10) at a maximum temperature of 40 °C under nitrogen stream. Add 1 ml of hydrolysis solution (5.11) and mix, followed by reaction at 40 °C for 1 h. Leave the solution at room temperature overnight. Then filter the solution using a PVDF membrane syringe filter (6.7).

### 8.2 HPLC analysis

#### 8.2.1 General

Inject 20 µl of the sample into the HPLC system (6.11). The first sample injected as part of a series of analysis shall be a blank of a methanol/water solution 80/20 v/v (5.7). There shall be no interfering signals present during the chromatographic run at the same retention time of hydroxytyrosol and tyrosol.

#### 8.2.2 HPLC conditions

The operating conditions given in [Table 1](#) have proven to be adapted for the determination.

2) The Spherisorb ODS2 column is an example of suitable chromatographic column that is commercially available. Alternative columns may be used, such as columns suitable for an Ultra-HPLC (UHPLC) system, if the operating conditions in [Table 1](#) are adjusted and the resulting precision data parameters are equal or better than those reported in [Tables C.3, C.4 and C.5](#). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

**Table 1 — Operating conditions**

Time min	Flow ml/min	A %	B %	C %
0	1,00	96	2	2
40	1,00	50	25	25
45	1,00	40	30	30
60	1,00	0	50	50
70	1,00	0	50	50
72	1,00	96	2	2
82	1,00	96	2	2

**Key**

A = water 0,2 % H<sub>3</sub>PO<sub>4</sub>  
 B = methanol  
 C = acetonitrile

The ternary gradient is programmed to enable an observation in the full chromatogram that hydrolysis is complete with no bound forms remaining in the extract. The chromatogram of the hydrolysed oil sample is compared with the chromatogram of the extract oil sample that has not been hydrolysed, which has been injected into the system under the same conditions, to determine whether hydrolysis is complete. Acetonitrile and methanol are always used in the same concentration ratio and therefore a binary system can also be used. Once the operators are experienced with the analysis, the elution time can be reduced by stopping the gradient after the elution of tyrosol, followed by washing the column for 10 min with B/C solvents in ratio of 50/50 v/v and then reconditioning for 10 min with A/B/C solvents 96/2/2 v/v/v.

The spectrophotometric detector (280 nm) shall be turned on an hour before the first analysis. The HPLC column shall be conditioned for at least 15 min before the gradient development with the initial solvent T = 0. Initially, inject 20 µl of external standard solution (5.10) and then, once the run is completed, inject 20 µl of the extracted sample solution (see 8.1).

Two independent determinations on the same sample shall be performed. The results shall respect repeatability values for a certain level of concentration. The result is the average of the two independent determinations.

At the end of the day, the system shall be conditioned with methanol/acetonitrile 50/50 v/v at a flow of 1 ml/min for at least 15 min. Store the chromatographic column with the end fitting closed.

An example of a chromatogram is shown in [Figure A.1](#).

### 8.2.3 Peak identification

Peak identification is carried out from the retention times by comparison with the external standard solution of known composition of hydroxytyrosol and tyrosol, which are eluted in the following order: hydroxytyrosol, tyrosol (see [Annex A](#)).

## 9 Expression of results

Calculate the areas of the two identified peaks through an electronic integrator, in mg/kg, using [Formulae \(1\)](#) and [\(2\)](#):

$$H = \frac{(A_{H\_sample}) \times 250 \times (C_H)}{(A_H) \times m} \quad (1)$$

where

$H$  is hydroxytyrosol content;

$A_{H\_sample}$  is the area corresponding to the peak of hydroxytyrosol of the sample recorded at 280 nm;

$A_H$  is the area corresponding to the peak of hydroxytyrosol of the external standard recorded at 280 nm;

250 is the multiplication factor used to express the result, in mg/kg, taking into consideration a final extraction volume of 5 ml and an injection volume of 20  $\mu$ l;

$C_H$  is the quantity, in  $\mu$ g, of hydroxytyrosol injected as external standard;

$m$  is the original sample mass, in grams.

$$T = \frac{(A_{T\_sample}) \times 250 \times (C_T)}{(A_T) \times m} \quad (2)$$

where

$T$  is tyrosol content;

$A_{T\_sample}$  is the area corresponding to the peak of tyrosol of the sample recorded at 280 nm;

$A_T$  is the area corresponding to the peak of tyrosol of the external standard recorded at 280 nm;

250 is the multiplication factor used to express the result, in mg/kg, taking into consideration a final extraction volume of 5 ml and an injection volume of 20  $\mu$ l;

$C_T$  is the quantity, in  $\mu$ g, of tyrosol injected as external standard;

$m$  is the original sample mass, in grams.

The total content is given by the sum of the content of the two components, as shown by [Formula \(3\)](#):

$$S = \Sigma (H + T) \quad (3)$$

where  $S$  is the total biophenolic compound contents.

Express this result without decimal digits.

Other peaks displayed in the chromatogram do not have to be considered.

## 10 Precision

### 10.1 Validation study

The details of the validation studies and the method precision are given in [Annex C](#). The obtained values cannot be applied to matrixes different from those indicated by the present method.

The repeatability values were obtained in accordance with ISO 5725-1<sup>[4]</sup>, ISO 5725-2<sup>[5]</sup>, ISO 5725-5<sup>[6]</sup> and ISO 5725-6<sup>[7]</sup> through a national validation study.

## 10.2 Repeatability, $r$

The absolute difference between two test results obtained by the same operator, with the same instruments and in the same operative conditions, on identical test material, over a short timescale shall not be over the  $r$  value for each concentration level in 95 % of cases.

## 10.3 Reproducibility, $R$

The difference between two single and independent results, obtained by different operators, in different laboratories, by using different equipment, on identical test material, shall not be over the  $r$  value for each concentration level in 95 % of cases.

## 11 Test report

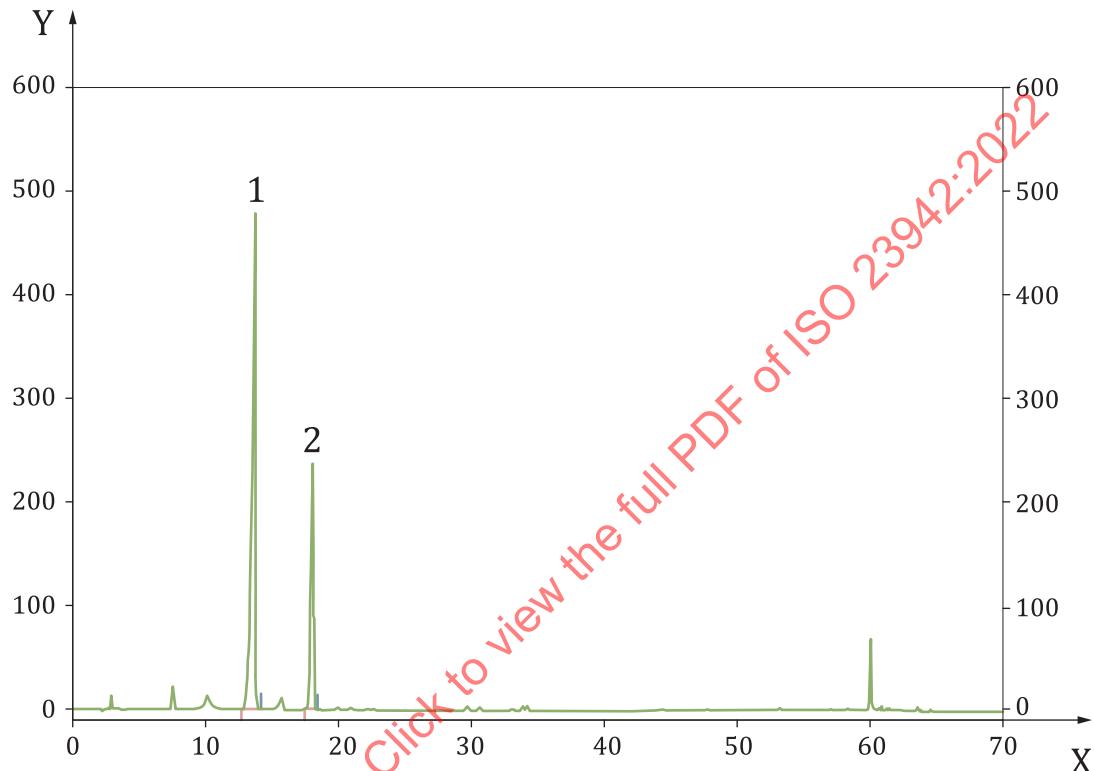
The test report shall specify:

- a) all information necessary for a correct sample identification;
- b) the sampling method used if known;
- c) the analysis method used in reference to this document, i.e. ISO 23942;
- d) all the operative details not specified in this document or regarded as optional, together with details of any incidents which can have influenced the test results;
- e) the results obtained in mg/kg of oil, of the single aromatic alcohols and of their sum, when over the limit of quantification (LOQ) value (in accordance with [Annex B](#)), as indicated by formulae reported in the method;
- f) any deviations from the procedure;
- g) any unusual features observed;
- h) the date of the test.

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## Annex A (informative)

### Chromatogram 280 nm

**Key**

- X time (in min)
- Y mV
- 1 hydroxytyrosol
- 2 tyrosol

**Figure A.1 — Example of a chromatogram**

## Annex B (normative)

### Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated on the basis of the ratio signal/noise (S/N) based on the peak determination area compared with the area of the noise. LOD and LOQ were calculated as the concentration of hydroxytyrosol and tyrosol spiked to a blank sample, without the presence of these aromatic alcohols that produce a recognizable peak from the noise with a ratio S/N of 3 (LOD) and 10 (LOQ).

The LOQ for hydroxytyrosol and tyrosol using the analytical condition of the present method shall be 1 mg/kg of oil (S/N 10), while the LOD shall be 0,5 mg/kg (S/N 3).

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## Annex C

### (informative)

## Validation studies

### C.1 First validation study

A collaborative study was conducted in Italy during the period April to May 2016, organized by INNOVHUB of Milan, with the participation of 10 national laboratories. The study was performed on five samples of extra virgin olive oils, pre-analysed for biophenols content (COI/T20/Doc. No 29<sup>[1]</sup> or NGD C89<sup>[2]</sup>). Each sample was analysed in duplicate. The results were evaluated in accordance with ISO 5725-1<sup>[4]</sup>, ISO 5725-2<sup>[5]</sup>, ISO 5725-5<sup>[6]</sup> and ISO 5725-6<sup>[7]</sup>. The statistical data are given in [Table C.1](#) after calculation with statistical software obtained from ARPAT (Regional Environmental Protection Agency in Tuscany). The anomalous values were determined by applying Cochran's and Grubbs' tests and those anomalous values were removed before calculating the Z-scores, which must be between -2 and +2 to be satisfactory.

**Table C.1 — Summary of statistical results — Sum of hydroxytyrosol and tyrosol**

Parameter	Samples				
	Extra virgin olive oil EU	Extra virgin olive oil EU	Extra virgin olive oil 100 % Italian	Extra virgin olive oil 100 % Italian	Extra virgin olive oil EU
Number of participating laboratories, $N$	10	10	10	10	10
Number of laboratories after anomalous elimination data, $n$	8	10	9	9	9
Mean, mg/kg	137,7	115,2	382,9	183,2	264,7
Median, mg/kg	137,5	112,5	379,0	184,0	259,5
Repeatability, $s_r$ , mg/kg	8,7	6,2	19,1	11,1	24,1
$C_{V,r}$ (%) ( $s_r \times 100/\text{mean}$ )	6,3	5,4	5,0	6,0	9,1
Repeatability limit, $r$ , mg/kg <sup>a</sup>	24,4	17,4	53,5	31,1	67,5
Reproducibility, $s_R$ , mg/kg	11,1	16,2	47,1	23,1	34,4
$C_{V,R}$ (%) ( $s_R \times 100/\text{mean}$ )	8,1	14,0	12,3	12,6	13,0
Reproducibility limit, $R$ , mg/kg <sup>b</sup>	31,1	45,4	131,9	64,7	96,3
Expanded Uncertainty, $Ue^c$	24,3	36,1	106,6	51,4	74,3
HorRat <sup>d</sup>	1,1	1,8	1,9	1,7	1,9

<sup>a</sup> Repeatability limit,  $r = 2,8 \times s_r$

<sup>b</sup> Reproducibility limit,  $R = 2,8 \times s_R$

<sup>c</sup> Expanded Uncertainty,  $Ue = t(n-1, p0,95) \times s_R$  where  $t$  = Student's  $t$ -test.

<sup>d</sup> HorRat value for the reproducibility,  $[C_{V,R \text{ exp}} / C_{V,R \text{ teor}}] = 2^{(1-0,5 \log C)}$  and  $C$  is the concentration expressed as 10 potency (Horwitz equation).

### C.2 Second validation study

A second collaborative study was coordinated by Italy during the period March to May 2021, organized by INNOVHUB of Milan, with the participation of 20 international laboratories. The study was performed

on five samples of extra virgin olive oils, pre-analysed for biophenols content (COI/T.20/Doc. No 29<sup>[1]</sup> or NGD C89<sup>[2]</sup>). Each sample was analysed in duplicate. The results were evaluated in accordance with ISO 5725-1<sup>[4]</sup>, ISO 5725-2<sup>[5]</sup>, ISO 5725-5<sup>[6]</sup> and ISO 5725-6<sup>[2]</sup>. The statistical data are given in [Tables C.3](#), [C.4](#) and [C.5](#) after calculation with statistical software obtained from ARPAT. The anomalous values were determined by applying Cochran's and Grubbs' tests and those anomalous values were removed before calculating the Z-scores, which must be between -2 and +2 to be satisfactory.

The samples used in the international collaborative trial are described in [Table C.2](#).

**Table C.2 — International collaborative trial samples**

Sample	Sample description
Extra virgin olive oil A	IGP Tuscany
Extra virgin olive oil B	Liguria 100 % Taggiasca
Extra virgin olive oil C	100 % Italian
Extra virgin olive oil D	100 % Italian
Extra virgin olive oil E	100 % Italian
Reference sample	Extra virgin olive oil Italian: 50 % Coratina; 30 % Ogliarola Garganica; 20 % Peranzana

**Table C.3 — Summary of statistical results — Hydroxytyrosol**

Parameter	Samples					Reference Sample
	Extra virgin olive oil A	Extra virgin olive oil B	Extra virgin olive oil C	Extra virgin olive oil D	Extra virgin olive oil E	
Number of participating laboratories, $N$	20	20	20	20	20	20
Number of laboratories after anomalous elimination data, $n$	16	18	17	16	18	16
Mean, mg/kg	252,6	7,2	51,6	107,4	87,4	257,7
Median, mg/kg	250,8	7,2	52,0	110,0	90,2	259,0
Repeatability, $s_r$ , mg/kg	4,8	1,1	3,0	4,3	4,9	7,0
$C_{V,r}$ (%) ( $s_r \times 100/\text{mean}$ )	1,9	15,0	5,7	4,0	5,6	2,7
Repeatability limit, $r$ , mg/kg <sup>a</sup>	13,4	3,1	8,4	12,0	13,7	19,6
Reproducibility, $s_R$ , mg/kg	19,3	2,0	7,2	10,0	12,0	13,6
$C_{V,R}$ (%) ( $s_R \times 100/\text{mean}$ )	7,6	27,6	14,0	9,3	13,7	5,3
Reproducibility limit, $R$ , mg/kg <sup>b</sup>	54,0	5,6	20,2	28,0	33,6	38,1
Expanded uncertainty, $Ue^c$	40,9	4,1	15,1	20,9	25,0	28,4
HorRat <sup>d</sup>	1,2	2,3 <sup>e</sup>	1,6	1,2	1,7	0,8

<sup>a</sup> Repeatability limit,  $r = 2,8 \times s_r$

<sup>b</sup> Reproducibility limit,  $R = 2,8 \times s_R$

<sup>c</sup> Expanded uncertainty,  $Ue = t(n-1, p0,95) \times s_R$  where  $t$  = Student's  $t$ -test.

<sup>d</sup> HorRat value for the reproducibility,  $[C_{V,R} \text{ exp} / C_{V,R} \text{ teor}] = 2^{(1-0,5 \log C)}$  and  $C$  is the concentration expressed as 10 potency (Horwitz equation).

<sup>e</sup> A content of 7,2 mg/kg is an unusually low concentration and therefore, a HorRat index of 2,32 is considered acceptable.