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Organo-mineral fertilizers - Identification of chelating agents - Part 2: Determination of Fe chelated by [o,o] EDDHA, [o,o] EDDHMA and HBED, or the amount of chelating agents by ion pair chromatography

Engrais organo-minéraux - Identification des agents chélatants - Partie 2 : Détermination du Fe chélaté par [o,o] EDDHA, [o,o] EDDHMA et HBED, ou de la quantité d'agents chélatants par chromatographie d'appariement d'ions

Organisch-mineralische Düngemittel - Identifizierung von Chelatbildnern - Teil 2: Bestimmung von Fe chelatisiert mit [o,o] EDDHA, [o,o] EDDHMA und HBED oder der Menge der Chelatbildner mittels Ionenpaarchromatographie

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European foreword

This document (CEN/TS 17789-2:2022) has been prepared by Technical Committee CEN/TC 260 “Fertilizers and liming materials”, the secretariat of which is held by DIN.

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Introduction

Micronutrients are considered to be, in plant nutrition, a number of elements known to be needed in small amounts for proper plant growth and development. The most common are Iron (Fe), Manganese (Mn), Molybdenum (Mo), Copper (Cu), Zinc (Zn) and Boron (B).

If an organo-mineral fertilizer contains a substance, or one of the substances in the mixture, which is intended to enhance the long term availability to plants of micronutrients in the EU fertilizing product, that substance is either a chelating agent or a complexing agent.

The chelating agents are divided into two groups¹:

- Group 1: EDTA, DTPA, HEEDTA, IDHA and [S,S]-EDDS;
- Group 2: Chelating agents present in UVCB (unknown or variable composition, complex reaction products or biological materials) chelates including [o,o] EDDHA, [o,p] EDDHA, [o,o] EDDHMA, HBED and EDDHSA.

This document defines the test method to be used in order to identify and determine the content of UVCB micronutrient chelated by [o,o] EDDHA, [o,o] EDDHMA and HBED in organo-mineral fertilizers (product function category (PFC) 1(B) according to Regulation (EU) 2019/1009 [4]).

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¹ Abbreviated terms are described in Annex A.

1 Scope

This document specifies a method for the determination by ion pair chromatography of the iron chelated by each individual *ortho*(hydroxy)-*ortho*(hydroxy) isomer of the chelating agents [o,o] EDDHA, [o,o] EDDHMA and by HBED in organo-mineral fertilizers, having an organic matrix based on vegetal residues (cocoa shells, grape residue, soybean residue, ...), algae extract, and animal meal (feather, bones, blood, ...) and containing one or more of these substances, except for [o,o] EDDHMA and HBED mixes.

The method allows the identification and the determination of the total concentration of water soluble iron chelates of these chelating agents. Also, after derivatization with Fe, the soluble amount of the chelating agents can be determined when other micronutrients beside Fe are present in organo-mineral fertilizers containing [o,o] EDDHA, [o,o] EDDHMA or HBED.

This method is applicable to a mass fraction of the metal chelated of at least 0,625 %.

NOTE 1 The substances EDDHA and EDDHMA exist as several different isomeric forms. Positional isomers for the hydroxyl or methyl groups (in *ortho*, *meta*, and *para* positions) as well as stereo isomers (*meso* and dl-racemic forms) are known. Both *meso* and dl-racemic forms of the [*ortho,ortho*] EDDHA and [*ortho,ortho*]. Since *para*, *meta* and *ortho* methyl positional isomers of the EDDHMA present quite similar stability, they could be grouped: in the method here described the *para*, *meta* and *ortho* methyl positional isomers of the [o,o] EDDHMA are considered together. HBED (N,N'-bis(2-hydroxybenzyl)-ethylenediamine-N,N'-diacetic acid) does not present isomeric forms.

NOTE 2 At present, analytically pure standards only exist for [*ortho,ortho*] EDDHA, [*ortho,ortho*] EDDHMA and HBED. All other substances being unavailable as a standard, the influence of their eventual presence in the samples (with respect to the sensitivity and the selectivity of this method) has not been studied.

NOTE 3 The *meso* and the dl-racemic forms of [o,o] EDDHA and [o,o] EDDHMA can be determined separately by this method.

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.

EN 12944-1, *Fertilizers and liming materials — Vocabulary — Part 1: General terms*

EN 12944-2, *Fertilizers and liming materials — Vocabulary — Part 2: Terms relating to fertilizers*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 12944-1 and EN 12944-2 apply.

ISO and IEC maintain terminological 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/>

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4 Principle

The iron chelates are separated and determined by isocratic ion-pair high-performance liquid chromatography. When an iron chelate (anion) is added to a polar fluid (eluent), containing a large cation, an ion pair is formed. This ion pair is retained by a non-polar solid phase (stationary phase). The strength of the retention depends on the molecular size and its acidity. Then, each iron chelate presents a characteristic retention time depending on the chelating agent, and it is separated from the other substances present in the sample. The separation is carried out on a reverse phase silica column and an aqueous solution of TBA⁺ (tetrabutylammonium) and acetonitrile as eluent. The detection is based on photometry at 280 nm.

For metal chelates different from Fe, a derivatization method may be used to form the Fe chelates, and then the chelating agent can be determined by the isocratic ion-pair high-performance liquid chromatography here presented.

5 Interferences

No interferences have been detected. Iron chelates with HBEP, EDDHSA, EDTA, DTPA, CDTA, HEEDTA, [p,p] EDDHA, [o,p] EDDHA, IDHA as well as the chelating agents do not interfere since they are separated from Fe-[o,o] EDDHA, Fe-[o,o] EDDHMA or Fe-HBED. Fe-[o,o] EDDHA does not interfere with Fe-[o,o] EDDHMA or Fe-HBED. In the case that Fe-[o,o] EDDHMA and Fe-HBED are present in the same sample, an overlapping of the meso isomer of the Fe-[o,o] EDDHMA and Fe-HBED peaks can occur with some equipment depending on the column used. The use of a different column of the same type can solve this problem.

6 Reagents

All reagents shall be of recognized analytical grade.

If products with a declared purity of less than 99 % are used for the preparation of standard solutions, a correction should be made in order to obtain exactly the required concentration in the solution.

If there is any doubt of the purity of the standard, it is necessary to determine it.

NOTE For this determination, a titrimetric method can be used. See Annex B for a general method using an automatic titrator. Manual titration could also be adequate.

6.1 Water.

All water used should conform to EN ISO 3696, be degassed and be free of organic contaminants.

6.2 Sodium hydroxide solution, substance concentration $c(\text{NaOH}) = 0,1 \text{ mol/l}$.

Dissolve 4 g of NaOH in pellet form in a 1 l volumetric flask with water (6.1). Dilute to the mark and homogenize.

The incorporation of CO₂ from the atmosphere should be carefully avoided. Otherwise, the dissolution of chelating agents (see 6.6, 6.7 and 6.8) can be incomplete.

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

Dilute 88 ml of hydrochloric acid (mass fraction 35 % HCl) to 1 000 ml with water (6.1).

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

Dilute 50 ml of hydrochloric acid 1,0 mol/l (6.3) to 500 ml with water (6.1).

6.5 Iron-nitrate solution, mass concentration $\rho(\text{Fe}) = 1\ 050 \text{ mg/l}$.

Dissolve 0,759 4 g of ferric nitrate 9-hydrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$) in 100 ml of water. Check (for example by AAS) that the Fe concentration in this solution amounts $1\,050 \text{ mg/l} \pm 30 \text{ mg/l}$.

NOTE As the $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ is deliquescent it will be added in solution of a known concentration.

6.6 Fe-[o,o] EDDHA solution, $\rho(\text{Fe}) = 100 \text{ mg/l}$.

Depending on the availability, either 6.6.1 or 6.6.2 procedures can be used to prepare the standard stock solution.

6.6.1 Preparation from Fe-[o,o] EDDHA.

Dissolve 5/SP g (where SP is the purity of the solid standard in percentage of Fe chelated), in 50 ml of water (6.1) in a 100 ml beaker and make up to 500 ml in a volumetric flask with water. The standard obtained in this way may be stored in darkness for one year.

6.6.2 Preparation from [o,o] EDDHA.

Dissolve 0,322 1 g (see 6.1) of ethylenediamine- N,N' -di[(*ortho*-hydroxyphenyl)acetic acid] in 350 ml of water (6.1) and 27 ml of NaOH (6.2) in a 500 ml beaker. Add 50 ml of the Fe solution (6.5) to the chelating agent solution, stirring for about 5 min.

Adjust the solution to pH 7,0 with NaOH solution (6.2). Let the solution stand overnight in darkness to allow excess Fe to precipitate as oxide. Filter quantitatively through a cellulose filter and make up to 500 ml in a volumetric flask with water (6.1). The standard obtained in this way may be stored in darkness for one year.

6.7 Fe-[o,o] EDDHMA solution, $\rho(\text{Fe}) = 100 \text{ mg/l}$.

Dissolve 0,347 1 g (see 6.1) of ethylenediamine- N,N' -di[(*ortho*-hydroxy-*para*-methylphenyl)acetic acid] [the *paramethyl* isomer of [o,o] EDDHMA, (see 1, Note 1)] in 350 ml of water (6.1) and 27 ml of NaOH (6.2) in a 500 ml beaker. Add 50 ml of the Fe solution (6.5) to the chelating agent solution, stirring for about 5 min.

Adjust the solution to pH 7,0 with NaOH solution (6.2). Let the solution stand overnight in darkness to allow excess Fe to precipitate as oxide. Filter quantitatively through a cellulose filter and make up to 500 ml in a volumetric flask with water (6.1). The standard obtained in this way may be stored in darkness for one year.

6.8 Fe-HBED solution, $\rho(\text{Fe}) = 100 \text{ mg/l}$.

Dissolve 0,347 1 g (see 6.1) of N,N' -bis(2-hydroxybenzyl)-ethylenediamine- N,N' -diacetic acid (HBED) or 0,379 7 g if HBED·HCl is used in 350 ml of water (6.1) and 27 ml of NaOH (6.2) in a 500 ml beaker. Add 50 ml of the Fe solution (6.5) to the chelating agent solution, stirring for about 5 min.

Adjust the solution to pH 5,0 with HCl solution (6.4) or NaOH solution (6.2). Let the solution stand overnight in darkness to allow excess Fe to precipitate as oxide. Filter quantitatively through a cellulose filter and make up to 500 ml in a volumetric flask with water (6.1). The standard obtained in this way may be stored in darkness for one year.

6.9 Eluent for the determination.

Add 20 ml of TBAOH (mass fraction 40 % Tetrabutylammonium hydroxide solution in water) to 600 ml of water (6.1). Adjust to pH 6,0 with hydrochloric acid solution (6.3 and 6.4). Add 300 ml of acetonitrile (HPLC grade) and make up to volume in a 1 l volumetric flask with water. Filter through a $0,2 \mu\text{m}$ membrane filter (7.4 b) and degas.

TBACl (Tetrabutylammonium chloride) or TBABr (Tetrabutylammonium bromide) may be used, providing that pH is adjusted to 6,0 with NaOH or HCl.

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7 Apparatus

Usual laboratory equipment, glassware and the following:

7.1 Magnetic stirrer.

7.2 Chromatograph, equipped with:

- a) an isocratic pump delivering the eluent at a flow rate of 1,5 ml/min;
- b) an injection valve with a 20 µl injection loop;
- c) a C-18 column; internal diameter: 3,9 mm; column length: 150 mm; particle diameter = 5 µm²;
- d) a C-18 guard column (recommended);
- e) a UV/VIS-detector with a 280 nm-filter;
- f) an integrator.

7.3 Balance, capable of weighing to the nearest 0,1 mg.

7.4 Membrane filters, including:

- a) micro membrane filters resistant to aqueous solutions, with porosity of 0,45 µm;
- b) micro membrane filters resistant to organic solutions (e.g. polyamide 66 micro membrane filters), with porosity of 0,2 µm.

8 Sampling and sample preparation

Sampling and sample preparation are not part of the method specified in this document. Recommended sampling methods are given in EN 1482-1 and, for sample preparation, in EN 1482-2.

For the size reduction of samples with a high amount of chelating agents, it is not recommended to use a high speed laboratory mill. It is more convenient to grind the sample to a particle size less than 1 mm with a mortar.

9 Procedure

9.1 Preparation of the sample solution for iron chelates

Weigh, to the nearest 0,1 mg, 200 mg of the iron chelate into a 250 ml beaker. Add 200 ml of water (6.1). Stir using a magnetic stirrer for 1 h. Transfer quantitatively into a 250 ml volumetric flask. Dilute to the mark with water and homogenize.

For samples declaring more than 5 % of chelated Fe, use a 500 ml volumetric flask.

For liquid samples, weigh an amount equivalent to 200 mg of solid.

² SYMMETRY™ C18, from WATERS, LiChroCART® Purospher® RP-18, from MERCK or equivalent are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of these products.

9.2 Preparation of the sample solution for other micronutrient chelates

Weigh, to the nearest 0,1 mg, 1 g of the chelate into a 250 ml beaker. Add 200 ml of water (6.1). Stir using a magnetic stirrer for 1 h. Transfer quantitatively into a 250 ml volumetric flask. Dilute to the mark with water (6.1) and homogenize. Filter through a paper filter. Pipette 20 ml of the solution into a 50 ml beaker. Add 10 ml of the iron(III) solution (6.5), homogenize, and allow to stand for 15 min. Adjust the pH to $9,0 \pm 0,1$ with sodium hydroxide solution (6.2) and allow to stand for another 15 min. Transfer quantitatively into a 100 ml volumetric flask. Dilute to the mark with water (6.1) and homogenize.

For samples declaring more than 5 % of chelated metals, use in the first step a 500 ml volumetric flask.

For liquid samples, weigh an amount equivalent to 200 mg of solid.

9.3 Preparation of the calibration solutions

Pipette V ml (see Table 1) of the Fe-[o,o] EDDHA (6.6) or Fe-[o,o] EDDHMA (6.7) or Fe-HBED (6.8) standard solution into six 100 ml volumetric flasks. Make up to volume with water (6.1) and homogenize.

Table 1 — Composition of the calibration solutions

Solution	V ml	Fe concentration in the <i>racemic</i> chelate mg/l	Fe concentration in the <i>meso</i> chelate mg/l	Total Fe concentration in chelate mg/l
1	5	2,5	2,5	5,0
2	10	5,0	5,0	10,0
3	20	10,0	10,0	20,0
4	30	15,0	15,0	30,0
5	40	20,0	20,0	40,0
6	50	25,0	25,0	50,0

Racemic and *meso* are optical isomers of the chelating agents [o,o] EDDHA and [o,o] EDDHMA. As the standard chelates solutions should be 50 % meso- and 50 % dl-racemic optical isomers, the concentration of the total iron chelate is the sum of the concentrations given in Table 1.

NOTE Since HBED does not present optical isomers the total concentrations can be used.

9.4 Chromatographic analysis

Immediately before injection, all solutions shall be filtered through a 0,45 μ m membrane filter [7.4]. Inject the calibration solutions (see 9.3) into the chromatographic system (7.2). Measure the retention times and the areas of the two Fe-[o,o] EDDHA or Fe-[o,o] EDDHMA isomers (*meso* and dl-racemic) or Fe-HBED peaks for all solutions. For each appropriate iron chelate, draw two calibration graphs with the values of the peak areas of the calibration solutions versus the iron concentration (mg/l) in the form of the corresponding chelating agent isomer. For Fe-HBED, only one graph is needed. See Figures 1 to 4.

NOTE See 9.2.

A statistically significant negative intercept can be caused by the contamination of the column with Fe oxide precipitates. In that case, the column should be replaced or cleaned.

Inject the sample solution (see 9.1 or 9.2). Identify the chelating agent by the retention time of the obtained peaks (see Figures 2, 3 and 4). Measure the areas of the isomer peaks for each chelating agent.