BS ISO 16308:2014

BSI Standards Publication

Water quality — Determination of glyphosate and AMPA — Method using high performance liquid chromatography (HPLC) with tandem mass spectrometric detection

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National foreword

This British Standard is the UK implementation of ISO 16308:2014.

The UK participation in its preparation was entrusted to Technical Committee EH/3/2, Physical chemical and biochemical methods.

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

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ISBN 978 0 580 71830 4

ICS 13.060.50

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 September 2014.

Amendments issued since publication

Date	Text affected

BS ISO 16308:2014

INTERNATIONAL STANDARD

ISO 16308

First edition
2014-09-15

Water quality — Determination of glyphosate and AMPA — Method using high performance liquid chromatography (HPLC) with tandem mass spectrometric detection

Qualité de l'eau — Détermination du glyphosate et de l'AMPA — Méthode par chromatographie en phase liquide à haute performance (CLHP) avec détection par spectrométrie de masse en tandem

Reference number
ISO 16308:2014(E)

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Published in Switzerland

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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).

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).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical and biochemical methods*.

Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] is a non-selective broad-spectrum herbicide. The efficiency of this compound makes it a top selling and one of the most widely used herbicides in the world since it entered the market in 1974. Together with its main degradation product, aminomethylphosphonic acid (AMPA), glyphosate is one of the most detected substances in water bodies in many developed countries. Note also that AMPA can be released during sewage treatment, e.g. due to breakdown of detergent formulations for textiles.

Glyphosate and AMPA belong to the aminophosphonate family and have specific physico-chemical properties that require the development of complex analytical methods for analysis and detection. The difficulty in analysis is mainly linked to the high solubility of glyphosate and AMPA and their chelating nature. To solve these problems, their pre-column derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl) to form less polar derivatives allows a better separation using liquid chromatography.

Gluphosinate, another aminophosphonate, is less commonly subject to regulation and can be determined simultaneously, provided it can be demonstrated that there is no interference with the sample under analysis.

There is currently an International Standard for the determination by liquid chromatography and fluorometric detection; however, the determination by HPLC–ESI–MS/MS can be much more specific (unambiguous identification) and more sensitive (limits of quantification of approximately 30 ng/l for both glyphosate and AMPA). This International Standard is based on this analytical technique and is intended for laboratories involved in the regulatory control of the aquatic environment. Many such laboratories are now equipped with this kind of apparatus.

INTERNATIONAL STANDARD ISO 16308:2014(E)

Water quality — Determination of glyphosate and AMPA — Method using high performance liquid chromatography (HPLC) with tandem mass spectrometric detection

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this International Standard be carried out by suitably qualified staff.

1 Scope

This International Standard specifies a method for the determination of dissolved fraction of glyphosate and its major metabolite, aminomethylphosphonic acid (AMPA), in drinking water, ground water, and surface water at concentrations of 0,03 µg/l to 1,5 µg/l. It does not apply to marine or salty water. This method can be applicable to other types of waters, provided the method is validated for each case.

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.

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

ISO 5667-3, *Water quality — Sampling — Part 3: Preservation and handling of water samples*

ISO 8466-1, *Water quality — Calibration and evaluation of analytical methods and estimation of performance characteristics — Part 1: Statistical evaluation of the linear calibration function*

3 Principle

Glyphosate and AMPA (dissolved fraction after filtration) are derivatized using 9-fluorenylmethylchloroformate (FMOC-Cl) (5.17) to lower their polarity and increase the retention of compound in a separation on a reverse phase column (e.g. C18) as well as to improve the mass spectrometric detection. If the mass spectrometer has sufficient detection capability, it is possible to omit the solid phase extraction and to analyse the analytes by direct injection (see 8.2.1).

The derivatized sample is purified by liquid/liquid extraction and then concentrated by solid phase extraction (SPE).

The analysis is performed by high performance liquid chromatography coupled with tandem mass spectrometry via an electrospray source (HPLC–ESI–MS/MS), using matrix-matched calibration.

Table 1 — Substances addressed

Name	Formula	Molecular mass g/mol	CAS-RN[a]
Glyphosate *N*-(phosphonomethyl)glycine	$C_3H_8NO_5P$	169,1	1071–83–6
AMPA Aminomethylphosphonic acid	CH_6NO_3P	111,0	1066–51–9
[a] CAS-RN Chemical Abstracts Service Registry Number			

NOTE Gluphosinate, belonging to the aminophosphonate family, can be determined simultaneously, provided it can be demonstrated that there is no interference with the sample (matrix) subject to analysis.

4 Interferences

This method is validated for hard water containing up to 3,2 mmol/l of the sum of calcium and magnesium. For waters with a higher calcium and magnesium content, it may be necessary to increase the concentration of EDTA-Na_2 (5.16) at the derivatization step (see Annex D).

It can prove necessary to include the acidification step described in Annex D even for some water types below 3,2 mmol/l of the sum of calcium and magnesium. The laboratory shall check the necessity of this procedure for its routine samples.

The presence of free chlorine, e.g. in treated waters, can cause losses of glyphosate by oxidation. Consequently sodium thiosulfate shall be used (see Clause 7).

5 Reagents

Unless otherwise stated, all reagents and solvents shall be of sufficient purity, e.g. “for trace analysis”.

5.1 Deionized water.

5.2 Ultra-pure water, complying with grade 1 of ISO 3696.

5.3 Nitrogen, N_2, purity ≥ 99,996 % volume fraction.

5.4 Laboratory detergent, alkaline.

5.5 Sodium thiosulfate, $Na_2S_2O_3$.

5.6 Acetonitrile, C_2H_3N, HPLC grade.

5.7 Methanol, CH_4O, HPLC grade.

5.8 Ethanol, C_2H_6O, 95 %, HPLC grade mass fraction.

5.9 Ethyl acetate, $C_4H_8O_2$, HPLC grade.

5.10 Ammonium acetate, $C_2H_7O_2N$.

5.11 Triethylamine, $C_6H_{15}N$.

5.12 Ammonium hydroxide, NH_4OH, 28 % mass fraction.

5.13 Formic acid, CH_2O_2.

5.14 Hydrochloric acid, HCl, 300 g/l.

5.15 Glacial acetic acid, $C_2H_4O_2$.

5.16 Ethylenediaminetetraacetic acid (EDTA), disodium salt dihydrate, $C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$, with a minimum purity of 99 % mass fraction.

5.17 9-Fluorenylmethyl chloroformate,(FMOC-Cl), $C_{15}H_{11}ClO_2$, with a minimum purity of 97 % mass fraction.

FMOC-Cl is used to prepare the **derivatization reagent**, FMOC-Cl solution, 50 mg/ml, in acetonitrile (5.6). This solution can be stored at −18 °C ± 3 °C for 6 months.

For direct injection (8.2.1), use a FMOC-Cl solution of 0,5 mg/ml in acetonitrile.

5.18 Reference substances, according to Table 1.

5.18.1 Glyphosate, *N*-(phosphonomethyl)glycine, $C_3H_8NO_5P$, purity > 98 % mass fraction.

5.18.2 AMPA, aminomethylphosphonic acid, CH_6NO_3P, purity > 98 % mass fraction.

5.18.3 1,2-$^{13}C_2$,^{15}N-labelled glyphosate, surrogate standard, purity > 98 % mass fraction.

5.18.4 ^{13}C,^{15}N-labelled AMPA, surrogate standard, purity > 98 % mass fraction.

5.19 Calibration solutions.

Individual stock solutions of glyphosate (5.18.1) and AMPA (5.18.2), 100 mg/l, prepared in ultra-pure water (5.2). These solutions can be stored at 4 °C ± 3 °C for 1 month.

Individual stock solutions of 1,2-$^{13}C_2$,^{15}N-labelled glyphosate (5.18.3) and ^{13}C,^{15}N-labelled AMPA (5.18.4) 100 mg/l, prepared in ultra-pure water (5.2). These solutions can be stored at 4 °C ± 3 °C for 1 month.

Multi-substance working solution of surrogates: 1,2-$^{13}C_2$,^{15}N-labelled glyphosate and ^{13}C,^{15}N-labelled AMPA, 20 µg/l, prepared in ultra-pure water (5.2). This solution can be stored at 4 °C ± 3 °C for 1 month.

NOTE Stock and calibration solutions can be stored longer, provided the adequate justifications are given regarding stability.

5.20 Triethylammonium acetate buffer, 0,1 % triethylamine (5.11) solution adjusted to pH 9,5 with glacial acetic acid (5.15) (mobile phase).

5.21 Sodium tetraborate, decahydrate, $Na_2B_4O_7{\cdot}10H_2O$.

5.22 Borate-Na buffer, 0,05 mol/l; pH = 9,2.

Dissolve 19 ± 0,1 g of sodium tetraborate (5.21), decahydrate in 1 l of water (5.1). This solution can be stored for approximately 1 mo at 4 °C ± 3 °C.

5.23 Mineral water, containing less than 3,2 mmol/l divalent cations (Mg^{2+} and total Ca^{2+}), for preparing matrix-matched calibration.

6 Apparatus

The material or any parts that are likely come into contact with the sample shall be free from any residue that could cause unacceptable interference in the blanks.

Glass and plastics containers can be used for sampling and for all steps before derivatization. Glass vials (6.10) and glass test tubes (6.11) shall be used after the derivatization step.

6.1 Usual laboratory glassware, or apparatus and in particular the following.

6.2 Glass, polyethene (PE) or **polypropene (PP) bottles**, minimum 50 ml, for sampling.

6.3 Glass, polyethene (PE) or **polypropene (PP) syringe**, 50 ml, for sample filtration.

6.4 Single use filter for syringe, diameter 25 mm, with a hydrophilic membrane, 0,45 µm, e.g. from regenerated cellulose.

6.5 Glass, or **single use PE** or **PP conical bottomed tubes**, approximately 50 ml, for derivatization.

6.6 Micropipettes, adjustable from 100 µl to 500 µl.

6.7 pH-meter.

6.8 SPE cartridges, e.g. Oasis HLB®[1)] Waters, 60 mg, 3 ml, or equivalent.

6.9 Centrifugation device, capable of 6 500 m^{-1}.

6.10 Glass vials, suitable for autosampler, with caps and polytetrafluoroethene (PTFE) or silicon rubber septa.

6.11 Glass test tube, 15 ml or smaller.

6.12 Reversed phase column, e.g. XBridge C18®[1)] column [Waters, 50 mm × 2,1 mm internal diameter (i.d.) 2,5 µm, column] with guard column (Waters, 10 mm × 2,1 mm i.d. 2,5 µm).

A column whose stationary phase is alkali proof (pH 9 to pH 9,5) is highly recommended.

NOTE A Gemini®-NX[1)] column (Phenomenex) with similar dimensions is also suitable. Other columns can be used, provided the separation conditions are adjusted.

6.13 High performance liquid chromatograph (HPLC), consisting of 6.13.1 to 6.13.5.

6.13.1 Injector, manual or automated.

6.13.2 Gradient pump.

6.13.3 Thermoregulation oven, for HPLC column.

6.13.4 Mass spectrometer, with triple quadrupole analyser and an electrospray source.

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1) Oasis HLB®, XBridge C18® and Gemini®-NX 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 ISO of these products.

6.13.5 Data acquisition and processing system.

7 Sampling

If the sampling bottles are not for single use, rinse the sample bottles (6.2) with deionized water (5.1), then clean with a laboratory detergent (5.4). Rinse with water (5.1), then with ultra-pure water (5.2), and finally with 95 % ethanol (5.8).

Perform the sampling in accordance with ISO 5667-3 in these bottles (6.2) (approximately 50 ml).

For samples suspected of containing free chlorine, add about 2 mg of sodium thiosulfate (5.5) or any other chlorine reducing agent per 100 ml of sample.

Store the samples according to ISO 5667-3 at 3 °C ± 2 °C and pre-treat within 24 h.

8 Procedure

8.1 Pre-treatment (Suspended particular matter)

Place filter (6.4) on syringe (6.3). Rinse with 5 ml of ultra-pure water (5.2).

Filter the sample (approximately 50 ml), discard the first 5 ml. Collect the filtrate in a conical bottomed tube (6.5).

Store the filtered samples at 4 °C ± 3 °C for one week maximum before the derivatization step.

8.2 Chelate break and derivatization

8.2.1 General

Adapt the derivatization step to the kind analytical step which follows derivatization:

— using a mass spectrometer with sufficient detection capability, no pre-concentration is needed and the derivatized sample is injected directly after derivatization according to 8.2.2;

— otherwise, a pre-concentration step can be useful to reach the intended limit of quantification (LOQ) performance, and derivatization shall be performed according to 8.2.3.

8.2.2 Chelate break and derivatization for direct injection

Place, e.g. 5 ml of the sample (different sample amounts requires the use of the equivalent amounts of the following reagents) in a 25 ml Erlenmeyer flask with glass stopper, add the magnetic stir bar.

Add 50 µl of a 20 µg/l multi-substances aqueous solution (5.19) of 1,2-$^{13}C_2$,^{15}N-labelled glyphosate (5.18.3) and ^{13}C,^{15}N-labelled AMPA (5.18.4) in ultra-pure water (5.2) as derivatization surrogate.

Add 100 µl EDTA-Na_2 (5.16) [0,1 mol/l solution in ultra-pure water (5.2)], shake, and allow to stand for 10 min in the closed flask.

Add, e.g. 2 ml of the borate-Na buffer (5.22) 0,05 mol/l and stir for 30 min at 400 min^{-1} to adjust to pH 9,0 to pH 9,5.

Add 400 µl of FMOC-Cl solution (5.17 for direct injection); stir for 4 h with 400 min^{-1} and wait for approx. 12 h (overnight).

Neutralize the solution with 30 % hydrochloric acid (5.14).

Filter and use for analysis (8.4).

The final volume of the pre-treated sample shall be considered when calculating the final result.

NOTE Valve switching of the eluate flow behind the column before and after passing of the analytes into the waste is a good choice to protect the ion source of the MS/MS detector from contamination.

8.2.3 Chelate break and derivatization prior to pre-concentration

Use, e.g. a micropipette (6.6), PP or PE pipette, place 5 ml sample in, for example, a conical bottomed PP tube (6.5).

Add 50 µl of a 20 µg/l multi-substances aqueous solution of 1,2-$^{13}C_2$,^{15}N-labelled glyphosate (5.18.3) and ^{13}C,^{15}N-labelled AMPA (5.18.4) in ultra-pure water (5.2) as derivatization surrogate.

Add 325 µl of borate-Na buffer (5.22) and shake.

Add 200 µl EDTA-Na_2 (5.16) [0,1 mol/l solution in ultra-pure water (5.2)], shake and allow to stand for 5 min. Addition of EDTA allows glyphosate and AMPA to be released from the complexes with divalent cations (e.g. Ca^{2+}, Mg^{2+}).

Add 4,5 ml acetonitrile (5.6). Shake well for 1 min to homogenize (there shall be no phase separation during this step of the procedure).

Add 0,6 ml of FMOC-Cl solution (5.17) and shake again.

Allow to stand for 30 min in the dark at room temperature (20 °C to 25 °C) for reaction.

After derivatization, excess FMOC-Cl and reaction byproducts (e.g. FMOC-OH) are removed during pre-concentration (8.3).

If the water is hard, $c(CaCO_3)$ > 3 mmol/l, an additional sample pre-treatment is recommended (see Annex E).

8.3 Pre-concentration

8.3.1 Liquid/liquid extraction of analytes derivates

Concentrate the analytical sample to approximately 5 ml under a nitrogen flow (20 ml/min to 40 ml/min) at room temperature to remove acetonitrile. The evaporation of the acetonitrile shall be completed and should not exceed 60 min. A FMOC-Cl (reagent excess) and FMOC-OH (byproduct) precipitate may crystallize on the tube wall.

Transfer the solution from the plastic tube in a glass test tube (6.11). Rinse the plastic tube with approximately 500 µl of ultra-pure water (5.2) and transfer into the glass tube.

Extract three times with 1,5 ml of ethyl acetate (5.9). If necessary, centrifuge for 20 s at 6 500 m^{-1} after each extraction to separate the two phases. Eliminate the supernatant with a Pasteur pipette (use a new pipette for each extraction).

Then concentrate the aqueous phase under a nitrogen flow for 15 min to evaporate the remaining ethyl acetate, shaking the tube every 5 min.

The final volume shall be approximately 6 ml.

The extract is then acidified to pH 3 with formic acid (5.13) to prevent any possible reaction with the residual FMOC-Cl and to allow the further pre-concentration of the extracts with SPE (8.3.2). For this purpose, add 100 µl of a 5 % volume fraction solution of formic acid (5.13) in ultra-pure water (5.2), adjust the volume to 6 ml with ultra-pure water (5.2) if necessary, and then homogenize for 1 min.

8.3.2 Solid phase extraction

8.3.2.1 General requirements

Use SPE cartridges (6.8) containing, e.g. 60 mg of 60 µm polystyrene-divinylbenzene polymeric solid phase. The recovery shall be checked on a regular basis.

NOTE 1 Oasis HLB®[1)] (60 µm, 60 mg, 3 ml, Waters), or equivalent may be used.

NOTE 2 Online SPE purification and concentration is allowed.

8.3.2.2 Cartridges conditioning

Rinse with 2 × 500 µl of methanol (5.7) and with 2 × 500 µl aqueous solution of 0,1 % volume fraction formic acid (5.13).

8.3.2.3 Purification

Pass 6 ml of acidified aqueous extract (pH 3) through the cartridge with an approximately 1 ml/min flow rate.

Rinse the SPE tube with 1 ml of an aqueous 0,1 % volume fraction solution of formic acid (5.13), then with 2 × 500 µl ultra-pure water (5.2).

Dry cartridge under vacuum or under a gentle stream of nitrogen during approximately 1 min to remove the main part of the water.

Elute with 3 × 700 µl of methanol (5.7): 20 g/l ammonium hydroxide (5.12) in ultra-pure water (5.2) (70:30 volume fraction).

Collect the eluate in a glass test tube (6.11) and reduce under a nitrogen flow until the methanol is removed. Final volume shall be approximately 0,7 ml.

NOTE Residues of methanol can cause degradation in chromatographic conditions, resulting in split peaks.

Adjust to 1 ml with ultra-pure water (5.2). The purified extract can be stored for 48 h at 4 °C ± 3 °C maximum prior to analysis due to the stability of the FMOC derivatives in water.

8.4 Chromatographic determination

8.4.1 General requirements

Use equipment in accordance with the instructions provided by the manufacturer.

Check the required system stability regularly. Adjust and optimize instrument parameter settings in accordance with the manufacturer's instructions.

Due to the use of a buffered mobile phase, it is recommended that the analytical column and the chromatographic system be rinsed according to the manufacturer's instructions on a regular basis.

8.4.2 Chromatographic conditions

Separate the glyphosate and AMPA derivatives by HPLC using a reversed phase column and appropriate chromatographic conditions. The pH of the mobile phase shall be alkaline (pH 9 to pH 9,5) in order to have better chromatographic performances (peak symmetry close to 1 and narrow peaks), which implies the use of a particular column (hybrid stationary phase).

Examples of appropriate chromatographic conditions are shown in Annexes B to D.

8.5 Identification and confirmation of the analytes

Identification of target compounds is achieved by comparing their retention time to the retention time of the referenced substance, and their single reaction monitoring (SRM) negative ionization mode mass spectra with the reference substances. Table 2 gives the characteristic transitions used.

Table 2 — ESI–MS/MS detection

Analytes	Quantifying transition	Qualifying transition
Glyphosate-FMOC	390 > 168	390 > 150
AMPA-FMOC	332 > 110	332 > 136
1,2-$^{13}C_2$,^{15}N-labelled glyphosate–FMOC	393 > 171	393 > 153
^{13}C,^{15}N-labelled AMPA–FMOC	334 > 112	334 > 138

The ratios between the quantifying and the qualifying transitions depend on the SRM conditions (i.e. collision energy and gas pressure in the collision cell). These ratios shall be determined using reference substances and checked for the samples to lie within ±20 % of the observed value for the reference substance, using the same equipment optimization.

Examples of chromatograms are given in Annex C.

8.6 Blank control monitoring

Blank controls are obtained with the application of procedure to a mineral water (5.23) spiked with 1,2-$^{13}C_2$,^{15}N-labelled glyphosate (5.18.3) and ^{13}C,^{15}N-labelled AMPA (5.18.4) only. Blank controls and samples shall be treated and analysed simultaneously. The residual content of both glyphosate and AMPA should be lower than 1/3 of the limits of quantification (LOQs).

9 Calibration

9.1 Concentration ranges

Prepare a five point calibration in accordance with ISO 8466-1 covering the working range from 0,03 µg/l to 1,5 µg/l for both glyphosate and AMPA, as well as for 1,2-$^{13}C_2$,^{15}N-labelled glyphosate (5.18.3) and ^{13}C,^{15}N-labelled AMPA (5.18.4) if a matrix matched calibration is carried out.

NOTE For routine analysis, a two point recalibration is sufficient.

9.2 Matrix-matched calibration

9.2.1 Calibration

Prepare a matrix-matched calibration applying procedure (see Clause 8) used for samples, to a mineral water (5.23) spiked with glyphosate (5.18.1), 1,2-$^{13}C_2$,^{15}N-labelled glyphosate (5.18.3), AMPA (5.18.2) and ^{13}C,^{15}N-labelled AMPA (5.18.4) according to 9.1. The matrix-matched calibration can be prepared with a real sample free of any trace of glyphosate and AMPA (residual concentrations lower than the LOQs).

Process calibration solutions like samples and establish a calibration function for each compound.

Determine the linear calibration function from (y_{ie}, ρ_{ie}) data pairs of the measurement series using Formula (1):

$$y_{ie} = m_i \rho_{ie} + b_i \quad (1)$$

where

y_{ie} is the measured response, expressed in units depending on the analytical method, e.g. area value, for a given ρ_{ie} of substance i in the calibration;

ρ_{ie} is the mass concentration of substance *i* in the calibration solution, in micrograms per litre, µg/l;

m_i is the slope of the calibration curve, in litres per microgram, l/µg;

b_i is the ordinate intercept of the calibration, expressed in units depending on the analytical method, e.g. area value.

1,2-$^{13}C_2$,^{15}N-labelled glyphosate and ^{13}C,^{15}N-labelled AMPA calibrations shall be used to determine surrogates in samples. The ratio of each surrogate in the sample versus calibration spiked solutions shall be used to monitor extraction and derivatization steps. (see Clause 10).

NOTE The slopes of the 1,2-$^{13}C_2$,^{15}N-labelled glyphosate and ^{13}C,^{15}N-labelled AMPA calibration curves can differ from the slopes of glyphosate and AMPA calibration curves, respectively. The differences of sensitivity between labelled and non-labelled standards depend on the instrumental configuration (geometry of the electrospray source, voltages, etc.).

9.2.2 Calculation

Determine mass concentration, ρ_{ig}, of substance *i* in sample using Formula (2):

$$\rho_{ig} = \frac{\left(y_{ig} - b_i\right)}{m_i} \quad (2)$$

where

ρ_{ig} is the mass concentration of substance *i* in the sample, in micrograms per litre, µg/l;

y_{ig} is the area value of chromatographic peak of substance *i* in the sample;

m_i,b_i see Formula (1).

The recovery of the surrogates after derivatization and HPLC-ESI-MS/MS analysis shall be 100 % ± 25 % for 1,2-$^{13}C_2$,^{15}N-labelled glyphosate-FMOC and ^{13}C,^{15}N-labelled AMPA-FMOC (determined with the corresponding matrix calibration curve, respectively).

9.3 Internal standard calibration

9.3.1 Calibration

Prepare an internal standard calibration applying the same procedure (see Clause 8) used for samples, to a mineral water (5.23) spiked with both glyphosate and AMPA (5.18), according to 9.1. Prior to the derivatization step, add 50 µl of a 20 µg/l multi-substances aqueous solution (5.19) of 1,2-$^{13}C_2$,^{15}N labelled glyphosate (5.18.3) and ^{13}C,^{15}N-labelled AMPA (5.18.4) in ultra-pure water (5.2).

The internal standard calibration can be prepared with a real sample free of any trace of glyphosate and AMPA (residual concentrations lower than the LOQs).

Process calibration solutions like samples and establish a calibration function for each compound.

Determine the linear calibration function [see Formula (3)] from pairs (y_{ie}, ρ_{ie}) of the measurement series using Formula (2).

9.3.2 Calculation

Establish a calibration function for each compound before each analytical series.

Plot the measured values, $y_{ie}/y_{int,ie}$ (peak areas, height of peaks or integration units, depending on the situation), for each substance *i* versus the corresponding mass concentration, $\rho_{ie}/\rho_{int,ie}$.

Determine the linear calibration function [see Formula (2)] with the help of the value pairs, $y_{ie}/y_{int,ie}$, and $\rho_{ie}/\rho_{int,ie}$ of the measurement series:

$$\frac{y_{ie}}{y_{int,ie}} = a_i \frac{\rho_{ie}}{\rho_{int,ie}} + b_i \quad (3)$$

where

y_{ie} is the measured response of substance *i* obtained from the calibration, dependent on ρ_{ie} — the unit depends on the evaluation, e.g. area values;

ρ_{ie} is the mass concentration of substance *i* in micrograms per litre, µg/l;

a_i is the slope of the calibration curve of substance *i*, e.g. area values in litres per microgram, l/µg;

b_i is the ordinate intercept of the calibration curve — the unit depends on the evaluation, e.g. area value;

$y_{int,ie}$ is the measured response of internal standard for the substance i dependent on $\rho_{int,ie}$ — the unit depends on the evaluation, area values for example;

$\rho_{int,ie}$ is the mass concentration of internal standard for the substance i in micrograms per litre, µg/l.

10 Expression of results

Report the results for substances listed in Table 1 in micrograms per litre, µg/l, to two significant figures.

EXAMPLE AMPA 0,85 µg/l Glyphosate 1,7 µg/l

11 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard (i.e. ISO 16308:2014);

b) identification of the sample;

c) the sample storage and pre-treatment procedure;

d) the results obtained for the individual substances, expressed in accordance with Clause 10;

e) any deviation from the procedure specified and of all circumstances that may have influenced the results.

Annex A
(informative)

Performance data

A.1 Performance data

An international interlaboratory trial was organized in April 2013. 11 laboratories from 3 countries took part.

Glyphosate and AMPA were analysed in

a) river water (sample 1),

b) drinking water (sample 2), and

c) underground water (sample 3).

The results summarized in Table A.1. were obtained by computing the raw data according to ISO 5725-2.

Table A.1 — Performance data

Sample	Substance	l	n	o	$\bar{\bar{x}}$	ρ_{ref}	η	s_R	$C_{V,R}$	s_r	$C_{V,r}$
				%	µg/l	µg/l	%	µg/l	%	µg/l	%
1	Glyphosate	11	44	0	0,666	0,705	94,5	115,3	17,3	39,4	5,9
	AMPA	11	44	0	0,807	0,836	96,5	115,5	14,3	42,9	5,3
	Gluphosinate	7	28	0	0,664	0,840	79,0	268,1	40,4	56,7	8,5
2	Glyphosate	10	40	10 *	0,049	0,042	116	0,013	26,0	0,004 8	9,8
	AMPA	9	36	11 *	0,056	0,047	119	0,020	35,6	0,004 8	8,6
	Gluphosinate	6	24	0	0,061	0,054	113	0,015	24,2	0,009 1	15,0
3	Glyphosate	11	44	0	0,151	0,142	106	0,043	28,3	0,007	4,4
	AMPA	11	44	0	0,149	0,129	116	0,039	26,3	0,011	7,1
	Gluphosinate	7	28	0	0,164	0,161	102	0,046	27,8	0,011	6,7

l number of laboratories after outlier rejection
n number of analytical results after outlier rejection
o percentage of outliers (*: one participant (4 data) excluded due to high blank value)
$\bar{\bar{x}}$ total mean of all results
ρ_{ref} reference value
η recovery
s_R reproducibility standard deviation
$C_{V,R}$ coefficient of variation of reproducibility
s_r repeatability standard deviation
$C_{V,r}$ coefficient of variation of repeatability

The samples used for this exercise were characterized for cations, especially those impairing hardness, as summarized in Table A.2.

Table A.2 — Matrix characterization

Elements	Blank	Spiked drinking water	Spiked under-ground water	Spiked surface water
Suspended matters (mg/l)	< 2	< 2	< 2	31,2
Chlorine (mg/l)	< 0,1	< 0,1	< 0,1	< 0,1
TOC (mg/l)	1,74	1,56	1,95	3,03
DOC (mg/l)	0,5	1,23	1,74	2,27
Ca^{2+} (mg/l)	81,2	115	92,9	116
Mg^{2+} (mg/l)	26,2	11,4	27,7	7,0

A.2 Performance data for direct injection method according to 8.2.2

These data were obtained with river water samples collected and analysed in Germany in 2013. (See Tables A.3 and A.4.)

3 river water samples were analysed according to 8.2.2. Sample 3 was analysed without modification; samples 1 and 2 were spiked to show concentrations distributed over the application range of the method. The matrices were characterized on ionic composition

Table A.3 — Matrix characterization for the German trial

Elements	Samples		
	Sample 1	Sample 2	Sample 3
	Rhein Düsseldorf Flehe	Ruhr Kahlenberg	Niers Kessel
pH	8,1	7,6	7,6
Conductivity (mS/m)	51	37	70
TOC (mg/l)	2,7	2,5	5,5
Cl^- (mg/l)	39,7	28,7	56
SO_4^{2-} (mg/l)	44,9	34,2	91
Na^+ (mg/l)	22	19	36
Ca^{2+} (mg/l)	59	37	83
Mg^{2+} (mg/l)	23	6,3	12
Fe (µg/l)	210	140	230
Mn^{2+} (mg/l)	23	35	39

Table A.4 — Performance data – direct injection

Sample	Substance	l	n	o	$\bar{\bar{x}}$	X_o	X_a	ρ_{ref}	η	s_R	$C_{V,R}$
				%	µg/l	µg/l	µg/l	µg/l	%	µg/l	%
sample 1 surface water	Glypho-sate	12	12	7,7	0,131	0,025	0,100	0,125	105	0,020	15,4
	AMPA	13	13	0	0,426	0,315	0,100	0,415	103	0,173	40,6
sample 2 surface water	Glypho-sate	11	11	8,3	0,193	0,025	0,150	0,175	110	0,029	15,2
	AMPA	11	11	18,1	0,646	0,501	0,150	0,651	99,2	0,043	6,6
sample 3 surface water	Glypho-sate	11	11	8,3	0,093	-	-	-	-	0,008	8,4
	AMPA	11	11	18,1	1,878	-	-	-	-	0,230	12,6

l is the number of laboratories after outlier rejection

n is the number of individual test results after outlier rejection

o is the percentage of outliers

X_o is the concentration in the original sample before spiking, analyzed in one laboratory

X_a is the spike value

ρ_{ref} is the reference value

$\bar{\bar{x}}$ is the overall mean of results (without outliers)

η is the recovery rate of the analyte

s_R is the reproducibility standard deviation

$C_{V,R}$ is the reproducibility variation coefficient

Annex B
(informative)

Examples of chromatographic conditions

HPLC: Ultimate 3000®[2] dual gradient micro [Dionex].

Mass spectrometer: API 2000™ [2] triple quadrupole mass spectrometer [AB Sciex].

Column: XBridge® C18 (50 mm × 2,1 mm i.d, 2,5 µm) (Waters) with guard (10 mm × 2,1 mm i.d, 2,5 µm). A Gemini NX® (Phenomenex) column with similar dimensions is also suitable.

Mobile phase: A (acetonitrile) and B (0,1 % volume fraction triethylamine buffer adjusted to pH 9,5 with acetic acid).

Flow rate: 400 µl/min

Oven temperature: 40 °C

Injection volume: 50 µl

Table B.1 — Analytical gradient

Time	A	B
min	%	%
0	8	92
1,5	8	92
3	95	5
4,5	95	5
6	8	92
11	8	92

Example conditions are given for an API 2000™ [2](Sciex):

Source: Turbo ion spray

Nebulisation gas GS 1: 207 kPa (30 psi)

Desolvation gas GS 2: 517 kPa (75 psi)

Curtain gas: 207 kPa (30 psi)

Collision gas CAD: 3

Scan mode: MRM

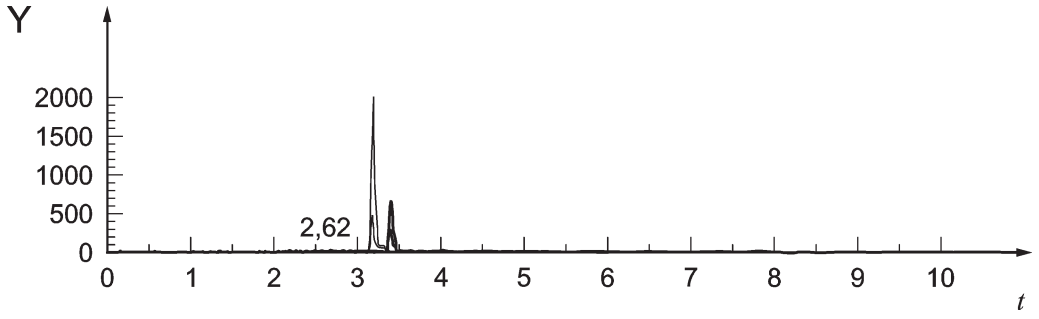
Source temperature: 450 °C

Ion spray volatege IS: −4,5 kV

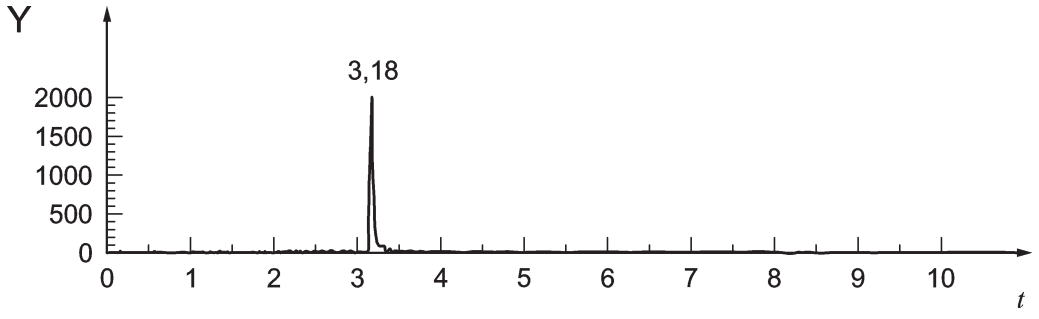
2) UltiMate 3000, Dionex API 2000™, XBridge®C18 and Gemini®-NX are examples of a suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Annex C
(informative)

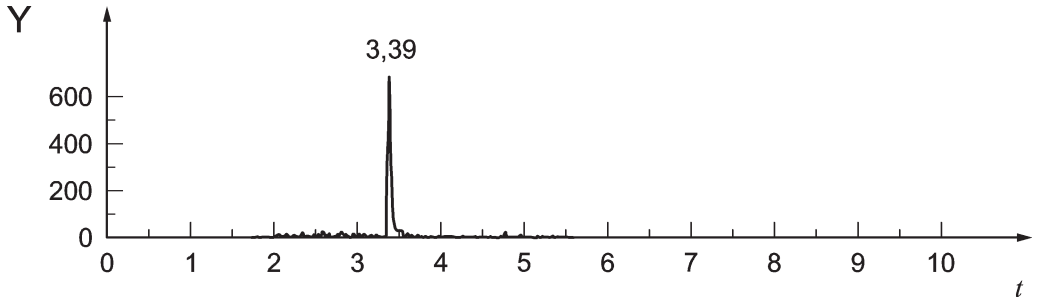
Examples of chromatograms



a) Glyphosate-FMOC and AMPA-FMOC standard mixture at 2 µg/l



b) 390 > 168 quantifying transitions for glyphosate-FMOC



c) 332 > 110 quantifying transitions for AMPA-FMOC

Key

Y abundance

t time

Figure C.1 — HPLC-ESI-MS/MS chromatogram

Annex D
(informative)

Analysis of gluphosinate

D.1 General

Gluphosinate [$C_5H_{12}NO_4P$, (*RS*)-2-amino-4-(hydroxymethylphosphinyl)butyric acid, CAS No. 51276-47-2] can be easily derivatized by FMOC-Cl together with glyphosate and AMPA. When using detection by LC-MS/MS, complete separation of compounds is not necessary.

At the time of publication, no gluphosinate isotope-labelled standard is available, thus external calibration or in case of the matrix leading to interference, a standard addition method is used for quantification.

D.2 HPLC — Method

D.2.1 Apparatus

Injection volume: 5 µl to 100 µl using a 120 µl sample loop.

Pre-column and analytical column: Nucleodur C18 Gravity®[3] Macherey-Nagel,[3] column dimensions: 8/3 mm and 125/2 mm, for example.

Oven temperature: 30 °C.

UV detector for control run at 225 nm.

Valve switching of flow before entering the MS (required to prevent contamination of ion source):

0 min to 5,5 min	waste;
5,5 min to 8,5 min	MS detector;
8,5 min to 20 min	waste.

Total run time: 20 min.

D.2.2 Reagents

Unless otherwise stated, all reagents and solvents shall be of sufficient purity, e.g. “for trace analysis”.

Eluent A, acetonitrile (5.6).

Eluent B, triethylamine buffer, pH 9,5.

3) Nucleodur C18 Gravity®, Macherey-Nagel and QTrap 4000® are examples of a 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.

Gradient:

Time, min	Composition, Eluent A, % volume fraction
0,5	5
11	95
12	5
14	5

D.3 Mass spectrometer conditions

Example conditions are given for a Qtrap 4000 ® (Sciex)[3].

Source parameters:

Source: Turbo ion spray

Nebulisation gas GS 1: 276 kPa (40 psi)

Desolvation gas GS 2: 414 kPa (60 psi)

Curtain gas: 172 kPa (25 psi)

Collision gas CAD: Medium

Scan mode: MRM

Source temperature: 350 °C [a]

Ion spray volatege IS: −4,5 kV

Interface heater IHE: on

Entrance potential EP: −10

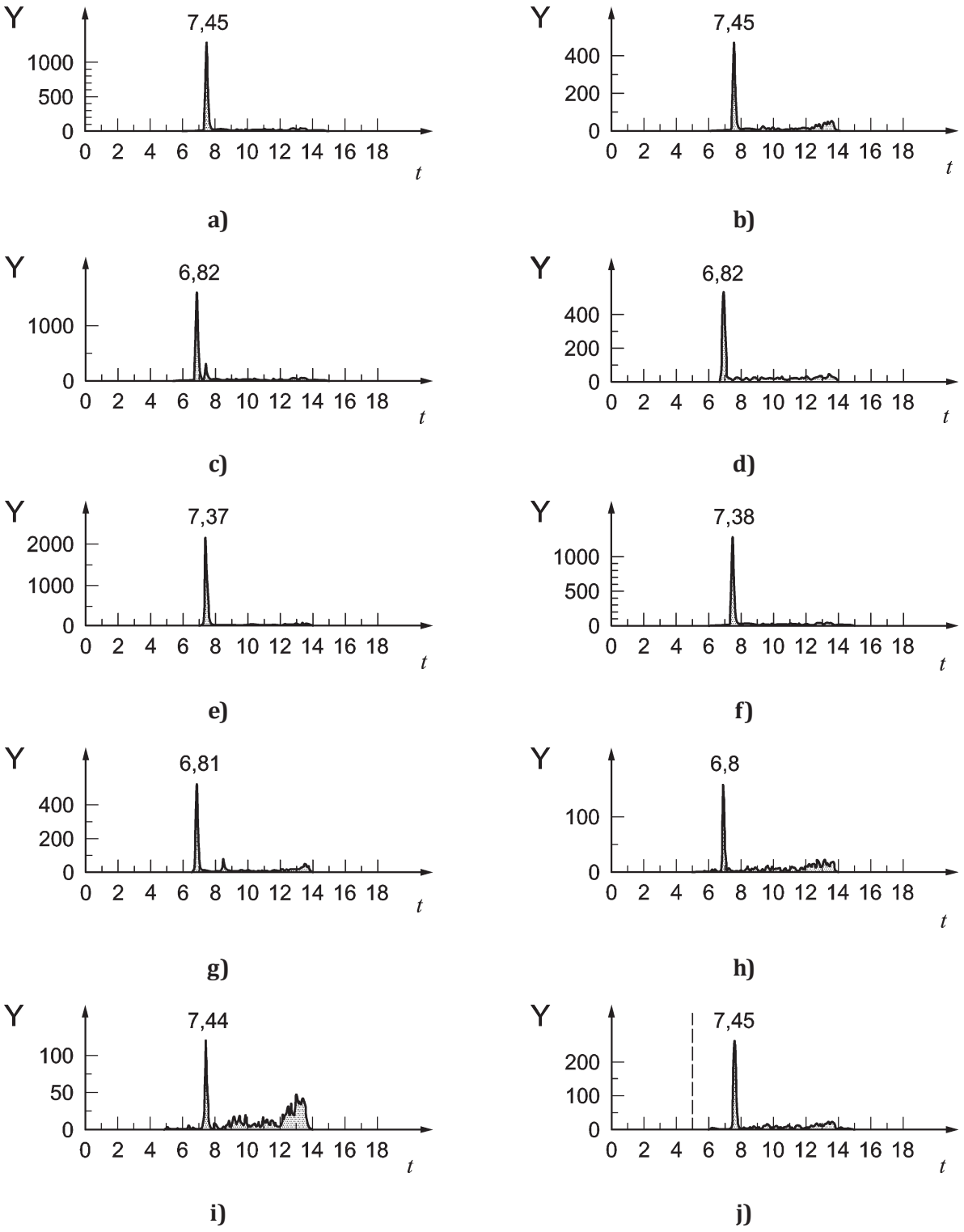
[a] At higher temperature, FMOC is split off.

Table D.1 — Example conditions for a QTrap 4000® (Sciex)[3]

Mass transitions (Q1 = quantifier, Q3 = qualifier)	**Q1 Mass** Da	**Q3 Mass** Da	**Dwell time** ms	**Parameter**	**Start**
AMPA–FMOC 1 [Shown in Figure D.1.a)]	332,000	109,900	50,00	DP	−60,00
				CE	−12,00
				CXP	−7,00
AMPA–FMOC 2 [Shown in Figure D.1 b)]	332,000	136,000	50,00	DP	−60,00
				CE	−20,00
				CXP	−1,00
Glyphosate–FMOC 1 [Shown in Figure D.1 c)]	390,100	167,700	50,00	DP	−54,00
				CE	−16,00
				CXP	−11,00

Table D.1 *(continued)*

Glyphosate–FMOC 2 [Shown in Figure D.1 d)]	390,100	150,000	50,00	DP	–54,00
				CE	–34,00
				CXP	–9,00
Glufosinate–FMOC 1 [Shown in Figure D.1 e)]	402,000	180,100	50,00	DP	–54,00
				CE	–14,00
				CXP	–5,00
Glufosinate–FMOC 2 [Shown in Figure D.1 f)]	402,000	205,900	50,00	DP	–54,00
				CE	–20,00
				CXP	–1,00
1,2-$^{13}C_2$,^{15}N-labelled glyphosate–FMOC 1 [Shown in Figure D.1 g)]	393,000	171,000	50,00	DP	–54,00
				CE	–16,00
				CXP	–11,00
1,2-$^{13}C_2$,^{15}N-labelled glyphosate–FMOC 2 [Shown in Figure D.1 h)]	393,000	153,000	50,00	DP	–54,00
				CE	–34,00
				CXP	–1,00
^{13}C,^{15}N-labelled AMPA–FMOC 1 [Shown in Figure D.1 i)]	334,000	138,000	50,00	DP	–60,00
				CE	–22,00
				CXP	–9,00
^{13}C,^{15}N-labelled AMPA–FMOC 2 [Shown in Figure D.1 j)]	334,000	112,000	50,00	DP	–60,00
				CE	–10,00
				CXP	–7,00
CE collision energy CXP collision cel exit potential DP declustering potential					



Key

Y abundance

t time (min)

Figure D.1 — Example of chromatograms

Annex E
(informative)

Pre-treatment of hard water samples

E.1 General

If the water hardness, measured as specified in ISO 6058[1] and/or ISO 6059[2], exceeds 3,2 mmol/l, a sample pre-treatment is recommended, in order to release any test substance chelated with divalent cations. Two preferred pre-treatment procedures are proposed (E.3 and E.4), the choice of which is left to the analyst.

It can prove be necessary to include this acidification step even for some water types below 3,2 mmol/l of the sum of calcium and magnesium. The laboratory shall check the necessity of this procedure for its routine samples.

E.2 Reagents

Unless otherwise stated, all reagents and solvents shall be of sufficient purity, e.g. "for trace analysis".

E.2.1 Hydrochloric acid, HCl, c(HCl) = 4 mol/l, quality "for trace analysis".

E.2.2 Oxalic acid dihydrate solution, $\rho(C_2H_2O_4 \cdot 2H_2O)$ = 100 g/l, CAS No. 6153-56-6.

Dissolve 50 g ± 0,05 g of oxalic acid dihydrate in 500 ml of water.

This solution can be stored at 4 °C ± 3 °C for about 2 months.

E.2.3 Potassium hydroxide solution, c(KOH) = 3 mol/l, CAS No. 1310-58-3.

Dissolve 16,8 g of potassium hydroxyde in 70 ml to 80 ml of water (5.1) in a 100 ml volumetric flask and make up with water.

This solution can be stored at 4 °C ± 3 °C for about 3 months.

E.3 First option for pre-treatment

Adjust 5 ml of sample to pH 1 with about 350 µl of hydrochloric acid (E.2.1).

Shake the mixture vigorously and wait for 1 min.

Adjust the sample to between pH 6 and pH 7 with potassium hydroxide solution (E.2.3).

Then proceed as specified in 8.2.

E.4 Second option for pre-treatment

Add 50 µl of oxalic acid dihydrate solution (E.2.2) to 5 ml of sample. Check that the pH lies between 2 and 3.

Shake the mixture vigorously and wait for 1 h.

Neutralize the sample with potassium hydroxide solution (E.2.3).

Then proceed as specified in 8.2.

Bibliography

[1] ISO 6058:1984, *Water quality — Determination of calcium content — EDTA titrimetric method*

[2] ISO 6059:1984, *Water quality — Determination of the sum of calcium and magnesium — EDTA titrimetric method*

[3] ISO 21458:2008, *Water quality — Determination of glyphosate and AMPA — Method using high performance liquid chromatography (HPLC) and fluorometric detection*

[4] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

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