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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

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Foreword

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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).

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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.

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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.

