

PETITORIO

00001

Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 07-087036- -00000-0000

Fecha: 2007-08-24 15:57:04 Dep. 2020 NUECREACION
Tra 11 PATENTE/II Eje: 378 FASENACIONAL
Act 411 PRESENTACION Folios 242

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FORMULARIO ÚNICO DE SOLICITUD DE PATENTE PCT ENTRADA EN FASE NACIONAL

①

SOLICITUD DE :

☒

Patente de Invención

☒

Capítulo I.

☐

Patente de Modelo de Utilidad

☐

Capítulo II.

②

SOLICITANTE
(71)

Nombre: BAYER CROPSCIENCE S.A.

Dirección: 16 rue Jean-Marie Leclair, F-69009 Lyon, Cedex 09, Francia

Nacionalidad o Domicilio: Lyon, Francia

Lugar de Constitución: Francia

Teléfono:

Fax:

E-Mail

IDENTIFICACION

C.C.

☐

NIT

☐

C.E.

☐

Otro

☐

Cual

Número

③

REPRESENTANTE
O APODERADO

Nombre: ALVARO CORREA ORDOÑEZ

Dirección: Avenida 82 No.10-62 Piso 6

Teléfono: 634 1500

Fax: 376 2211

E-Mail: office.bogota@bakernet.com

IDENTIFICACION

C.C.

☒

NIT

☐

C.E.

☐

Otro

☐

Cual

Número 79.143.366 de

Usaquén

TP 20.281

④

INVENTOR (ES)
(72)

Nombre: ROSATI, Dominique y VENET, Catherine

Dirección: 13, Chemin des Dares, F-01700 NEYRON, Francia y Le Colombier, F-69210 Saint Germain sur L'Arbrisle, respectivamente.

Nacionalidad o Domicilio: Francesa

⑤ PCT (85)

Solicitud Internacional No. PCT/EP2006/001433

Fecha 23-ENE-06

Publicación Internacional No. WO 2006/079566

Fecha 03-AGO-06

⑥ Título (54)

MÉTODO PARA ANALIZAR EL ÁCIDO FOSFOROSO, EL FOSETIL-AL, O AMBOS SIMULTÁNEAMENTE

⑦

Clasificación Internacional (51)

G01N 30/06, G01N 30/72

⑧

Prioridad

SI

☒

NO

☐

(33) País de Origen

EP

EP

EP

(32) Fecha

25-ENE-05

11-FEB-05

16-DIC-05

(31) Número de Solicitud

05356015.7

05356031.4

05356216.1

⑨

Comprobante de pago No.

7-66624

Fecha

16-AGO-07

10

ANEXOS

00002

- ☒ Comprobante de pago de la tasa de presentación de la solicitud
- ☒ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica
- ☒ Poderes, si fuere el caso
- ☐ Documento de cesión del inventor al solicitante o a su causante
- ☐ Declaraciones
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen preliminar (IPEA)
- ☐ Traducción del examen preliminar internacional efectuado por la IPEA
- ☐ De ser el caso, copia del contrato de acceso
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas
- ☐ De ser el caso, certificado de depósito del material biológico
- ☐ Arte final 12 x 12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud
- ☐ De ser el caso, modificaciones efectuadas a la solicitud internacional.
- ☒ Otros: Extratos, Reporte de Búsqueda, opinión escrita y notificación de presentación de documento de prioridad

11

FIGURA CARACTERISTICA

12

Solicito la concesión de la patente,

NOMBRE: ALVARO CORREA ORDOÑEZ

FIRMA:

C.C. 79.143.366 de Usaquén T.P. 20.281

Instrucciones para la presentación de los anexos, ver reverso de esta página

POD-02638

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 07-087036- -00000-0000

Fecha: 2007-08-24 15:57:04 Dep: 2020 NUECREACION:
Tra: 11 PATENTALIY Cve: 378 FASENACIONALI
Act: 411 INSUNTACION Folios: 242

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

HIT : 000176007-2

RECIBO OFICIAL DE CAJA : 07 50,000
FECHA : 2007-08-24

**** CONSIGNACION ****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	MT. PAGO
RAISBECK LARA RODRIGUEZ	CONSIGNACION	BANCO POPULAR	050-00110-6	48717720	16/08/2007	72,144,000.00

**** CONCEPTO ****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50205-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	482,000.00
		TOTAL :	\$ 482,000.00

CON: CUATROCIENTOS OCHENTA Y DOS MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Raisbeck, Lara, Rodríguez & Rueda
Av. 82 No. 10 - 62 Piso 6
Bogotá, Colombia

NIT: 860.068.840-3
Tel: +57 1 634 1500
Fax: +57 1 376 2211
www.bakernet.com

POWER OF ATTORNEY

The undersigned.

BAYER CROPSCIENCE SA

domiciled in

16, rue Jean-Marie Leclair
69009 LYON, France

declares that it hereby grants to

**ALVARO CORREA-ORDOÑEZ and/or OLGA
GEORGETTE OTERO and/or RICARDO METKE and/or
JUAN PABLO CONCHA.**

with domicile at Av. 82 No. 10 - 62, 6th floor, of Bogotá D. C., Colombia, a full and sufficient power of attorney to obtain the protection of my (our) industrial property and against the unfair competition, for which purpose I (we) authorize the said attorneys to represent me (us) before any authorities or persons, with or without jurisdiction, specially those administrative, administrative contentious and judicial, as well as before Arbitration Court, in all matters concerning the following: a) Obtainment of patents, utility models, industrial designs, integrated circuits, layout-designs and vegetal varieties; the registration of trademarks, denominations of origin, commercial names, trade emblems, slogans and domain names; its renewal, assignments, change of name or domicile, voluntary cancellation; and the registration of licenses of use of the above rights; b) Processes of protection to the consumer, unfair competition, oppositions, cancellations, nullity, precautionary injunctions, infringement, granting of licenses, writ mandamus, the protection of trade secrets, and in general the civil, penal, an administrative processes relating to those rights. And that in all the above matters they may substitute this Power of Attorney, compromise, conciliate, admit the facts of the case, desist, cancel, receive, resign, and ratify acts done by representatives without a power of attorney.

I hereby ratify all actions previously taken on behalf of this company by the aforementioned attorneys with respect to the mentioned matters.

PODER

La abajo firmada,

BAYER CROPSCIENCE SA

domiciliada en

16, rue Jean-Marie Leclair
69009 LYON, France

declara por el presente que otorga a

**ALVARO CORREA-ORDOÑEZ and/or OLGA GEORGETTE
OTERO and/or RICARDO METKE and/or JUAN PABLO
CONCHA.**

con domicilio en la Av. 82 No. 10 - 62 Piso 6 en Bogotá D. C., Colombia, poder especial amplio y suficiente, para obtener la protección de mi (nuestra) propiedad industrial y contra la competencia desleal, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera autoridades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, contencioso-administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de patentes de invención, modelos de utilidad, diseños industriales, esquemas de trazado de circuitos integrados y variedades vegetales; el registro de marcas, denominaciones de origen, nombres comerciales, enseñas, lemas comerciales y nombres de dominio; su renovación, traspaso, cambio de nombre o domicilio, cancelación voluntaria; y el registro de licencias de uso de esos derechos; b) Los procesos para la protección del consumidor, competencia desleal, oposiciones, cancelaciones, nulidades, medidas cautelares, infracciones, concesión de licencias, la tutela, la protección de secretos industriales, y en general procesos civiles, penales, administrativos relacionados con estos derechos. Y para que en todos los asuntos arriba determinados, puedan sustituir este mandato, transigir, conciliar, admitir los hechos del caso, desistir, cancelar, recibir, renunciar, y ratificar los actos de agentes oficiosos.

Igualmente, ratifico todo lo actuado hasta el momento en los mismos asuntos, por cualquiera de los mencionados apoderados en representación de ésta sociedad.

BAKER & MCKENZIE

Raisbeck, Lara, Rodríguez & Rueda
Av. 82 No. 10 - 62 Piso 6
Bogotá, Colombia

NIT: 860.068.840-3
Tel: +57 1 634 1500
Fax: +57 1 376 2211
www.bakernet.com

POWER OF ATTORNEY

The undersigned.

BAYER CROPSCIENCE SA

domiciled in

16, rue Jean-Marie Leclair
69009 LYON, France

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**ALVARO CORREA-ORDOÑEZ and/or OLGA
GEORGETTE OTERO and/or RICARDO METKE and/or
JUAN PABLO CONCHA.**

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I hereby ratify all actions previously taken on behalf of this company by the aforementioned attorneys with respect to the mentioned matters.

Done and subscribed in
Lyon

On the February 11th, 2005 day of

Alexander NOWAK



PODER

La abajo firmada,

BAYER CROPSCIENCE SA

domiciliada en

16, rue Jean-Marie Leclair
69009 LYON, France

declara por el presente que otorga a

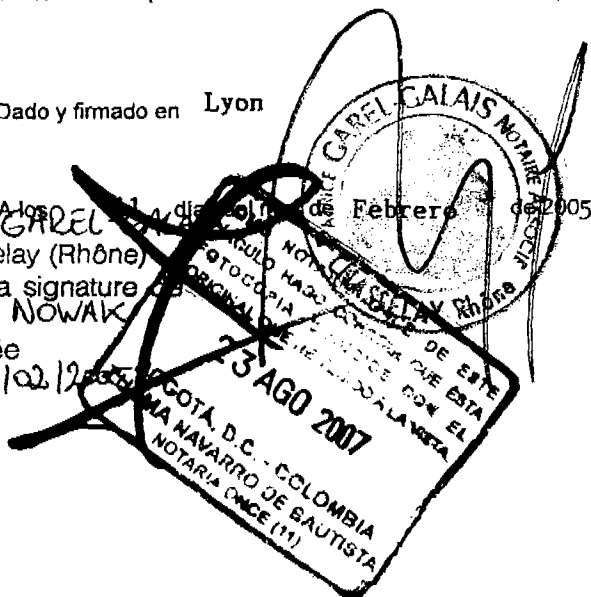
**ALVARO CORREA-ORDOÑEZ and/or OLGA GEORGETTE
OTERO and/or RICARDO METKE and/or JUAN PABLO
CONCHA.**

con domicilio en la Av. 82 No. 10 - 62 Piso 6 en Bogotá D. C., Colombia, poder especial amplio y suficiente, para obtener la protección de mi (nuestra) propiedad industrial y contra la competencia desleal, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera autoridades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, contencioso-administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de patentes de invención, modelos de utilidad, diseños industriales, esquemas de trazado de circuitos integrados y variedades vegetales; el registro de marcas, denominaciones de origen, nombres comerciales, enseñanzas, lemas comerciales y nombres de dominio; su renovación, traspaso, cambio de nombre o domicilio, cancelación voluntaria; y el registro de licencias de uso de esos derechos; b) Los procesos para la protección del consumidor, competencia desleal, oposiciones, cancelaciones, nulidades, medidas cautelares, infracciones, concesión de licencias, la tutela, la protección de secretos industriales, y en general procesos civiles, penales, administrativos relacionados con estos derechos. Y para que en todos los asuntos arriba determinados, puedan sustituir este mandato, transigir, conciliar, admitir los hechos del caso, desistir, cancelar, recibir, renunciar, y ratificar los actos de agentes oficiosos.

Igualmente, ratifico todo lo actuado hasta el momento en los mismos asuntos, por cualquiera de los mencionados apoderados en representación de ésta sociedad.

Dado y firmado en Lyon

Je soussigné M. GAREL, le 11 février 2005, à Chasselay (Rhône),
Notaire à Chasselay (Rhône)
Certifie sincère la signature de
MR Alexander NOWAK
qui a été apposée
Chasselay, le 11/02/2005



APOSTILLE

(Convention de La Haye du 5 octobre 1961)

Claudio

1. -- République française

Le greffier en chef public

2. -- a été substitué par *GAZEL GAUJIS Patrice*

3. -- a été substitué par *Nobre*

4. -- a été substitué par *CHASSELAU (22)*

Attesté

up le *16/10/05*

Par le Procureur général près la cour d'appel

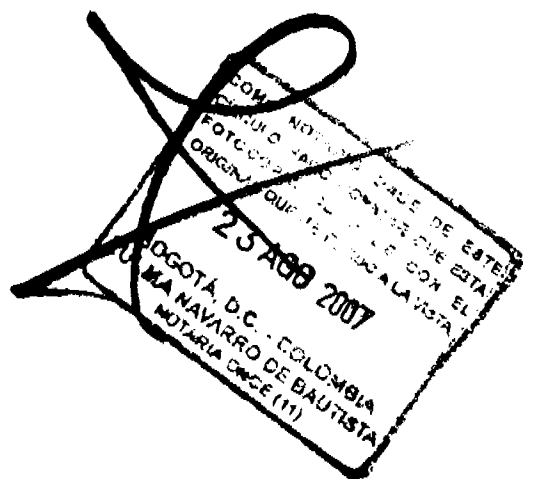
Sous le *273*

Château

10. -- Signature



[Signature]



NOTARIAL CERTIFICATION

On this _____ day of 10 Hars
2005, the undersigned Notary

CERTIFIES

1. That Alexander NOWAK
of legal age and domiciled in Neuenhouse Weg 10,
51467 Bergisch Gladbach, Germany
set his hand on the foregoing document.

2. That the grantor is to me personally known and that he/she has legal capacity to execute the instrument.

3. That the grantor has executed the foregoing document as
Authorized Representative

of the judicial person Bayer Crop Science S.A.

4. That the judicial person Bayer Crop Science S.A.
having its home office in 16, rue Jean-Marie Leclair
69009 LYON, France

is duly organized, has present legal existence and that the purposes for which the power of attorney is granted are within the scope of its corporate purposes.

5. That the grantor has in fact the referred to authority and that his/her representation is legal.

6. That I have based certifications 3, 4 and 5 above on the following authentic documents for such purpose exhibited to me and which I mention specifically, giving their dates and their origin or source:

-Page "annonces judiciaires et légales" from the newspaper "Le Tout Lyon-Moniteur Judiciaire Reunis" dated 21 to 27 June 2003

-Power of Attorney from Bayer Cropscience SA (from the Chairman of the Management Board) dated 15 October 2004


Notary Public

CERTIFICACION NOTARIAL

A los 10 días del mes de Marzo
de 2005, el suscrito Notario

DA FE

1. Que Alexander NOWAK
mayor y domiciliado en Neuenhouse Weg 10,
51467 Bergisch Gladbach, Alemania
suscribió de su puño y letra el documento que antecede.

2. Que conozco al otorgante y que éste tiene capacidad legal para el otorgamiento.

3. Que el otorgante ha suscrito el referido documento en su carácter de
Representante Autorizado

de la persona jurídica Bayer CropScience S.A.

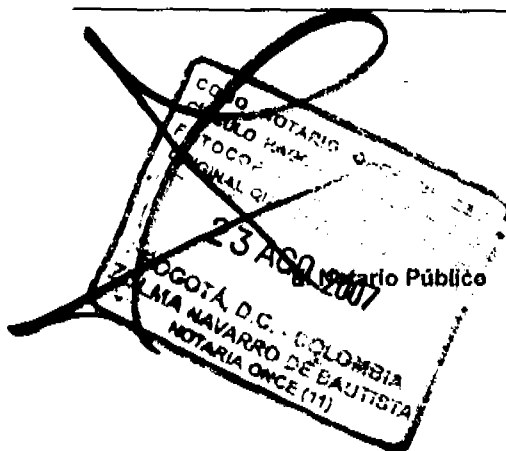
4. Que la persona jurídica Bayer CropScience S.A.

con sede en 16, rue Jean-Marie Leclair
69009 Lyon, Francia

está legalmente constituida, que tiene existencia legal actual y que el acto para el cual se otorga el poder está comprendido entre los que constituyen su objeto social.

5. Que el otorgante tiene efectivamente la representación que ostenta y que ésta es legítima.

6. Que he basado las certificaciones 3, 4 y 5 anteriores en los siguientes documentos auténticos que al efecto se me exhibieron y que menciono específicamente con expresión de sus fechas y de su origen o procedencia:



APOSTILLE

(Convention de La Haye du 5 octobre 1961)

.....COLONIE.....

1. — République française

Le présent acte public

2. — a été signé par BORÉL Gilbert Rédic

— agissant en qualité de Notaire

— est revêtu du sceau de son étude à

Châtelain (Houma)

Atteste

5. — à Mon 6. — le 13 Mars 2008

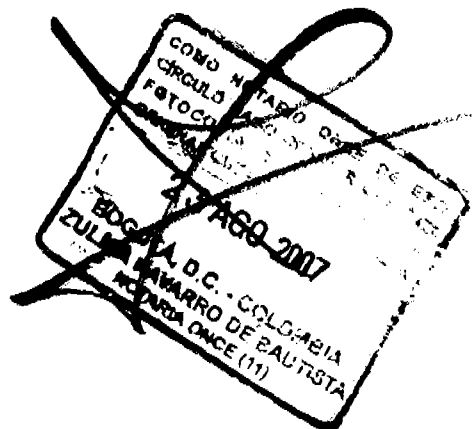
7. — Par le Procureur général près la Cour d'appel

8. — sous n° 1756

9. — Sceau 10. — Signature



[Handwritten signature]



(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 August 2006 (03.08.2006)

PCT

(10) International Publication Number
WO 2006/079566 A1

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G01N 30/06 (2006.01) G01N 30/72 (2006.01)

(21) International Application Number:
PCT/EP2006/001433

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05356015.7 25 January 2005 (25.01.2005) EP
05356031.4 11 February 2005 (11.02.2005) EP
05356216.1 16 December 2005 (16.12.2005) EP

(71) Applicant (for all designated States except US): **BAYER CROPSCIENCE SA** [FR/FR]; 16, rue Jean-Marie Leclair, F-69009 LYON CEDEX 09 (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ROSATI, Dominique** [FR/FR]; 5, Chemin des Dares, F-01700 NEYRON (FR). **VENET, Catherine** [FR/FR]; Le Colonnier, F-69210 Saint Germain sur L'Arbresle (FR).

(74) Agent: **BALMEFRESZOL, Ludovic**; Bayer CropScience SA, 14/20, rue Pierre Baizet, Boite Postal 9163, F-69263 Lyon Cedex 09 (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW). Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR). OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF ANALYZING PHOSPHOROUS ACID, FOSETYL-AL OR BOTH SIMULTANEOUSLY

(57) Abstract: Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 5 mg/kg of a sample, comprising the following steps: a) preparation of the sample; b) optional dilution of the sample prepared; c) direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC) /tandem mass spectrometry (MS/MS).

WO 2006/079566 A1

METHOD OF ANALYZING PHOSPHOROUS ACID, FOSETYL-AL
OR BOTH SIMULTANEOUSLY

DESCRIPTION

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The present invention relates to the field of pesticidal compound analysis. Such methods are useful for monitoring the behaviour of such pesticidal compounds once they have been applied, and such methods are also useful during marketing authority procedures for such pesticidal compounds.

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The compounds that can be analyzed by means of the method of analysis according to the invention are compounds that are useful for the protection of plants and also some of the compounds that are metabolites of these biologically active compounds. Some of these metabolites can also exhibit biological activities.

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Methods for analyzing pesticidal compounds are known. In particular, an analytical method for determining the fosetyl-Al residue and its main metabolite, phosphorous acid, in drinking water or in surface water is known.

20

Such a known method uses (trimethylsilyl)diazomethane (TMSD) as a derivatizing agent.

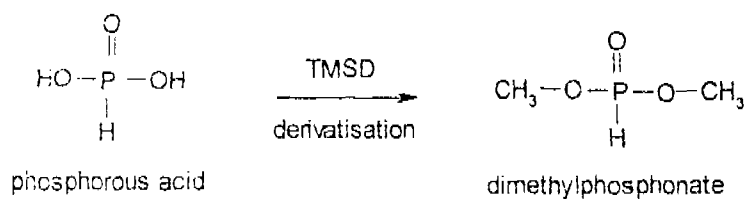
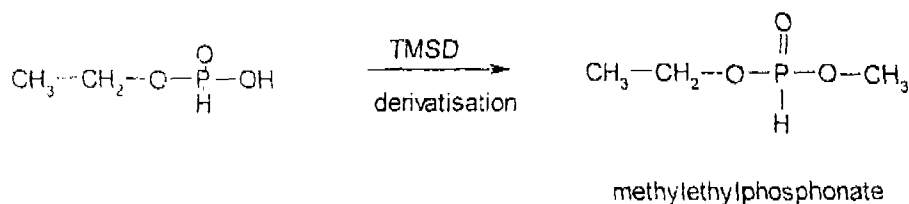
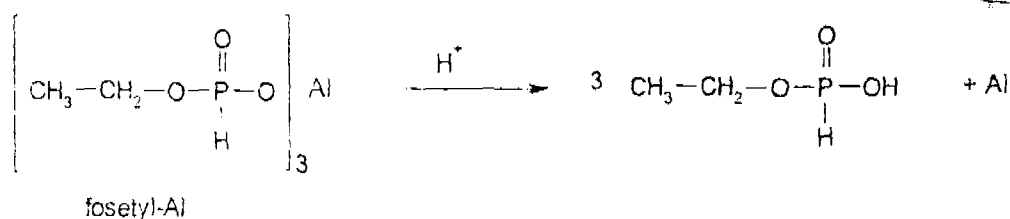
Generally, such a method comprises the following steps:

- concentration of the water samples ;
- derivatization with (trimethylsilyl)diazomethane of an aliquot of the concentrated
- 25 sample (substitution of a hydrogen atom with a methyl group) ;
- purification of the derivatized sample by liquid-liquid partition with dichloromethane.

30

The analysis is carried out by gas chromatography on a semi-capillary column by means of a flame photometric detector (or FPD) in the phosphorous mode and the quantification takes external standards as reference. The use of a thermionic detector is also possible.

This known method is carried out according to Scheme 1 below:



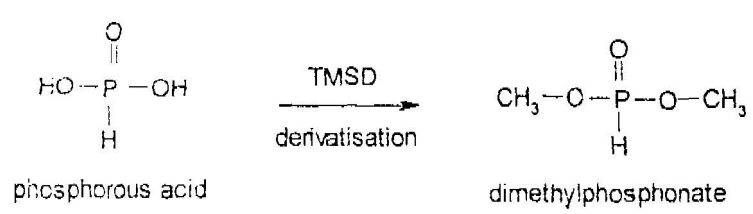
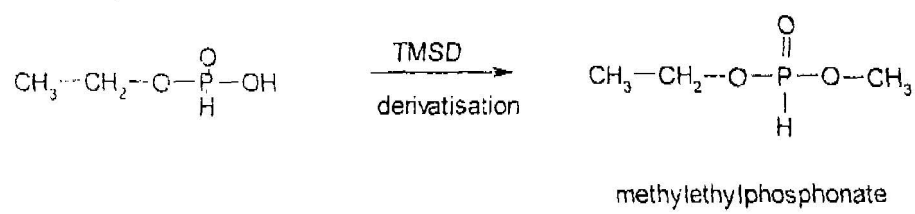
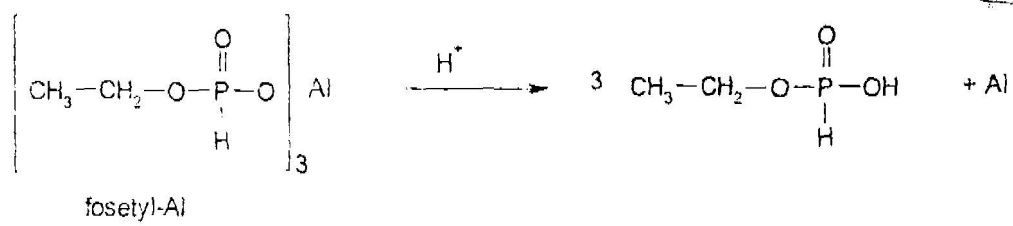
With this method, the limits of detection (LODs) are as follows:

- for fosetyl-Al:
 - 0.05 µg/l for drinking water (mineral or mains water) ;
 - 0.05 µg/l for surface water (river water) ;
- for phosphorous acid:
 - 0.7 µg/l for drinking water (mineral or mains water) ;
 - 2.5 µg/l for surface water (river water).

With this method, the limits of quantification (LOQs) are as follows:

- for fosetyl-Al:
 - 0.1 µg/l for drinking water (mineral or mains water) ;
 - 1 µg/l for surface water (river water) ;
- for phosphorous acid:
 - 2.0 (g/l for drinking water (mineral or mains water) ;
 - 4.0 (g/l for surface water (river water).

This known method of analysis has been used for the following substrates: mineral water, mains water and water from the Rhône river. The method of analysis has been validated for various types of water, by analysis of non treated control samples enriched in fosetyl-Al and in phosphorous acid to the limits of quantification and to 10 times these limits.



With this method, the limits of detection (LODs) are as follows:

- for fosetyl-Al:
 - 0.05 µg/l for drinking water (mineral or mains water) ;
 - 0.05 µg/l for surface water (river water) ;
- for phosphorous acid:
 - 0.7 µg/l for drinking water (mineral or mains water) ;
 - 2.5 µg/l for surface water (river water).

With this method, the limits of quantification (LOQs) are as follows:

- for fosetyl-Al:
 - 0.1 µg/l for drinking water (mineral or mains water) ;
 - 1 µg/l for surface water (river water) ;
- for phosphorous acid:
 - 2.0 (g/l for drinking water (mineral or mains water) ;
 - 4.0 (g/l for surface water (river water).

This known method of analysis has been used for the following substrates: mineral water, mains water and water from the Rhône river. The method of analysis has been validated for various types of water, by analysis of non treated control samples enriched in fosetyl-Al and in phosphorous acid to the limits of quantification and to 10 times these limits.

The analysis of these enriched samples gives recovery rate values for fosetyl-Al or for phosphorous acid, which are compared with the expected theoretical values.

As another known method of analysis, mention may be made of a method for analyzing fosetyl-Al or phosphorous acid residues present in soils taken in Chazay d'Azergues (France), in Goch (Germany) and in Seville (Spain).

This method itself also uses (trimethylsilyl)diazomethane. It is also carried out according to Scheme 1.

10

In this course of this method, the fosetyl-Al and phosphorous acid residues are extracted from the soil samples by agitation in the presence of an ammonia buffer solution, and then the cations that are present are removed from the extracts by means of an ion exchange resin and the water is evaporated from the samples. Finally, the extracts obtained are derivatized by the action of (trimethylsilyl)diazomethane.

15

Subsequently, the quantification is carried out by gas chromatography on a semi-capillary column using a flame photometric detector (in the phosphorous mode) with an external standard.

20

The limit of quantification (LOQ) of this method is 0.100 mg/kg for each of the compounds.

The control samples were enriched in fosetyl-Al or in phosphorous acid up to the limit of quantification and also up to 100 times this limit.

25

Another known method for analyzing residues concerns the analysis of fosetyl-Al and phosphorous acid residues in plant samples, derived both from fruits and from vegetables.

This method itself also uses (trimethylsilyl)diazomethane. It is also carried out according to Scheme 1.

30

In the course of this method, the fosetyl-Al and phosphorous acid residues are extracted from the plant samples by milling in a mixture of water and acetonitrile. The extracts are subsequently purified using a C18 cartridge and are then derivatized by the action of (trimethylsilyl)diazomethane.

35

Subsequently, the quantification is carried out by gas chromatography on a semi-capillary column using a flame photometric detector (in the phosphorous mode) with an external standard.

The analysis of these enriched samples gives recovery rate values for fosetyl-Al or for phosphorous acid, which are compared with the expected theoretical values.

As another known method of analysis, mention may be made of a method for analyzing fosetyl-Al or phosphorous acid residues present in soils taken in Chazay d'Azergues (France), in Goch (Germany) and in Seville (Spain).

This method itself also uses (trimethylsilyl)diazomethane. It is also carried out according to Scheme 1.

10

In this course of this method, the fosetyl-Al and phosphorous acid residues are extracted from the soil samples by agitation in the presence of an ammonia buffer solution, and then the cations that are present are removed from the extracts by means of an ion exchange resin and the water is evaporated from the samples. Finally, the extracts obtained are derivatized by the action of (trimethylsilyl)diazomethane.

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This method itself also uses (trimethylsilyl)diazomethane. It is also carried out according to Scheme 1.

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In the course of this method, the fosetyl-Al and phosphorous acid residues are extracted from the plant samples by milling in a mixture of water and acetonitrile. The extracts are subsequently purified using a C18 cartridge and are then derivatized by the action of (trimethylsilyl)diazomethane.

35

Subsequently, the quantification is carried out by gas chromatography on a semi-capillary column using a flame photometric detector (in the phosphorous mode) with an external standard.

The limit of quantification of this known method is 0.50 mg/kg for each of the products, with the exception of hop, for which this limit is 2.0 mg/kg for fosetyl-Al and 20.0 mg/kg for phosphorous acid.

5

This method has been used on samples of bunches of grapes, of oranges, of bananas, of strawberries, of lettuce and of cucumbers. The control samples were enriched, in particular up to the limit of quantification.

10 Yet another known method for analyzing residues concerns the analysis of fosetyl-Al or phosphorous acid residues in animal tissues or products of animal origin, such as milk, bovine meat, bovine kidneys, bovine liver or eggs.

15 According to this study, the residues of compounds to be analyzed are extracted from the samples by double milling in a water/acetonitrile mixture (50/50, 20/80 for milk).

An aliquot of the extract is subsequently purified by means of a C18 cartridge (except for milk). The purified extract is subsequently derivatized with a solution of TMSD.

20 This method of analysis also follows Scheme 1

The quantification is carried out by gas chromatography on a DB Wax column using a flame photometric detector in the phosphorous mode.

25 The limits of quantification are as follows:

- 0.50 mg/kg for fosetyl-Al and phosphorous acid in bovine meat, bovine kidney, bovine liver and eggs ;
- 0.10 mg/kg for fosetyl-Al and phosphorous acid in milk.

30 For this method, non treated control samples were prepared and analyzed, along with samples enriched to the limit of quantification and also to several times this limit.

The known methods of analysis that have just been mentioned are in accordance with the provisions of European directive No. 46 from 1996 (96/46/EC of 16 July 1996), in particular with
35 respect to the following characteristics:

- for each of the substrates and each level:
 - the mean of the recovery rates should be between 70 and 110% ;

The limit of quantification of this known method is 0.50 mg/kg for each of the products, with the exception of hop, for which this limit is 2.0 mg/kg for fosetyl-Al and 20.0 mg/kg for phosphorous acid.

5

This method has been used on samples of bunches of grapes, of oranges, of bananas, of strawberries, of lettuce and of cucumbers. The control samples were enriched, in particular up to the limit of quantification.

10 Yet another known method for analyzing residues concerns the analysis of fosetyl-Al or phosphorous acid residues in animal tissues or products of animal origin, such as milk, bovine meat, bovine kidneys, bovine liver or eggs.

15 According to this study, the residues of compounds to be analyzed are extracted from the samples by double milling in a water/acetonitrile mixture (50/50, 20/80 for milk).

An aliquot of the extract is subsequently purified by means of a C18 cartridge (except for milk). The purified extract is subsequently derivatized with a solution of TMSD.

20 This method of analysis also follows Scheme 1

The quantification is carried out by gas chromatography on a DB Wax column using a flame photometric detector in the phosphorous mode.

25 The limits of quantification are as follows:

- 0.50 mg/kg for fosetyl-Al and phosphorous acid in bovine meat, bovine kidney, bovine liver and eggs ;
- 0.10 mg/kg for fosetyl-Al and phosphorous acid in milk.

30 For this method, non treated control samples were prepared and analyzed, along with samples enriched to the limit of quantification and also to several times this limit.

The known methods of analysis that have just been mentioned are in accordance with the provisions of European directive No. 46 from 1996 (96/46/EC of 16 July 1996), in particular with
35 respect to the following characteristics:

- for each of the substrates and each level:
 - the mean of the recovery rates should be between 70 and 110% ;

- the repeatability, expressed as variation coefficient (ratio of the standard deviation to the mean for the sample concerned, expressed as a percentage) should be at most 20% ;
- for each of the substrates, the total variation coefficient (all levels included) should be at most 20%.

Another known method for analyzing fosetyl-Al is described in an article entitled Rapid determination of fosetyl-Al residues in lettuce by liquid chromatography/electrospray tandem mass spectrometry (Hernandez et al., Journal of AOAC International, Vol. 86, No. 4, 2003).

The method described concerns the quantification of fosetyl-Al residues in plant samples that are derived from lettuce. The method requires a step consisting of extraction with water by means of a high-speed mixer, followed by the injection of a 5-fold diluted extract into a liquid-phase chromatograph.

The fosetyl-Al is therefore quantified by liquid chromatography coupled to electrospray tandem mass spectrometry after addition of tetrabutylammonium acetate as an ionic pairing agent.

The analysis of samples of lettuce enriched at 2 and 0.2 mg/kg is reported. The limit of quantification is 0.2 mg/kg, whereas the limit of detection of fosetyl-Al is 0.05 mg/kg.

However, many of these known methods of analysis require a chemical derivatization step. Such an additional step complicates the analysis and very substantially prolongs the duration thereof. Furthermore, the implementation of this step requires specific expertise and increases the financial cost of these methods.

In addition, during such a derivatization step, the derivatizing agents used, which may be TMSD, diazomethane or other alternative derivatizing agents, are reactants which, in addition to their high cost, present considerable risks when they are used. Among the risks encountered when using such derivatizing agents, mention may be made of their toxicity and also their explosiveness. The use of such agents also results in a high cost.

Moreover, these known methods comprise many handling steps (evaporations, re-dissolutions, sample transfers) increasing, accordingly, the loss and dissemination of the compounds to be analyzed. Such a dissemination of compounds can also pose the problem of its environmental impact, in particular when the effluents derived from these methods of analysis are retreated.

Furthermore, these known methods have the major drawback of not being specific for particular compounds. This lack of specificity can result in compounds for which the protection and

quantification characteristics are similar, not being differentiated. Other known methods have the disadvantage of only allowing the analysis of fosetyl-Al alone, without being able to carry out the simultaneous analysis of phosphorous acid, for example.

- 5 Some of the known methods are described only for particular matrices ; for example, one already known method concerns only particular plant tissues derived from lettuce.

Finally, these known methods do not make it possible to achieve certain stricter limits of quantification, in particular the limits of quantification ensuing from recent regulations, for
10 example directive 96/46/EC of 16 July 1996.

A method of analysis has now been found which makes it possible to provide a solution to these problems or to prevent these drawbacks related to the known methods.

- 15 Thus, the present invention relates to a method for analyzing residues of pesticidal compounds.

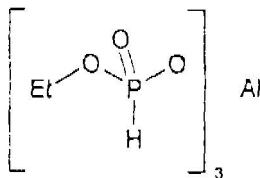
The method according to the invention may be suitable for the analysis of pesticidal compounds, whether they are fungicides, herbicides, insecticides or growth regulators.

- 20 Advantageously, the method of analysis according to the invention is used for analyzing residues of fungicidal compounds.

Particularly advantageously, the method of analysis according to the invention is used for analyzing fosetyl-Al residues and phosphorous acid residues.

25

fosetyl-Al is a fungicidal compound of phosphonic acid type, the chemical name of which is ethyl hydrogen phosphonic acid aluminium salt, having the formula



Phosphorous acid has the formula H_3PO_3 .

30

Thus, among other advantages, the method of analysis according to the present invention is of great simplicity. Furthermore, this method is direct and it makes it possible to achieve levels of quantification of the pesticidal compounds analyzed that have never before been achieved.

The method according to the invention is also particularly advantageous from an environmental point of view, and also economically.

In general, the present invention relates to a method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.00005 mg/kg, preferably less than or equal to 0.000005 mg/kg, more preferably less than or equal to 0.0000005 mg/kg of a sample, comprising the following steps:

- preparation of the sample ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

For the liquid samples analyzed according to the present invention, the limits of quantification can be expressed in mg/l. Those skilled in the art will be able to make the necessary conversions.

The method of analysis according to the invention may comprise a step consisting of dilution of the sample prepared.

The method of analysis according to the invention is suitable for the simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

Preferably, the method according to the invention is used for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ; phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or of one or more salts thereof ; more preferably for the analysis of fosetyl-Al or of phosphorous acid.

Particularly advantageously, the method of analysis according to the invention is used for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

Preferably, the method of analysis according to the invention can be used for the analysis of samples chosen from plant tissues, preferably a plant matrix with a high water content, a plant matrix with an acidic pH, a dry plant matrix, a fatty plant matrix ; water, preferably mineral water, underground water, mains water or surface water ; soils ; animal products or tissues, preferably milk, eggs, liver, kidneys, fats, muscle ; air ; agrofood products, preferably converted, and human body fluids such as blood and urine.

For the method of analysis according to the invention, the preparation step can be chosen from an extraction for plant tissues ; soils ; animal products or tissues and converted agrofood products ; optional concentration for water and trapping for air.

5 Such a concentration step may also be used for other samples.

For the method of analysis according to the invention, the dilution step can be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid, acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be
10 acidified ; or alternatively in a mixture of such solvents.

According to a first particular aspect of the invention, it relates to a method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.005 mg/kg, preferably less than or equal to 0.00005 mg/kg, more preferably less than or equal to 0.0000005 mg/kg of
15 a water sample, comprising the following steps:

- preparation of the water sample ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

20 The method for analyzing a water sample according to the invention may comprise a step consisting of dilution of the sample prepared.

The method for analyzing a water sample according to the invention is suitable for the
25 simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

Preferably, the method for analyzing a water sample according to the invention is used for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ;
30 phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or one of more salts thereof ; more preferably for the analysis of fosetyl-Al.

Particularly advantageously, the method for analyzing a water sample according to the invention is used for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

35 Preferably, the method for analyzing a water sample according to the invention can be used for the analysis of samples chosen from mineral water, underground water, mains water or surface water.

For the method for analyzing a water sample according to the invention the preparation step may be a concentration.

For the method for analyzing a water sample according to the invention, the dilution step may
5 be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid, acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be acidified ; or alternatively in a mixture of such solvents.

According to a second particular aspect of the invention, it relates to a method for analyzing one
10 or more pesticidal compounds present in amounts of less than or equal to 1 mg/kg, preferably less than or equal to 0.01 mg/kg, more particularly less than or equal to 0.001 mg/kg of a plant tissue sample, comprising the following steps:

- preparation of the plant tissue sample ;
- optional dilution of the sample prepared ;
- 15 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

The method for analyzing a plant tissue sample according to the invention may comprise a step
20 consisting of dilution of the sample prepared.

The method for analyzing a plant tissue sample according to the invention is suitable for the
25 simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

Preferably, the method for analyzing a plant tissue sample according to the invention is used for
the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ;
phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or one or more
salts thereof ; more preferably for the analysis of fosetyl-Al.

30 Particularly advantageously, the method for analyzing a plant tissue sample according to the invention is used for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

Preferably, the method for analyzing a plant tissue sample according to the invention can be
used for the analysis of samples chosen from a plant matrix with a high water content, a plant
35 matrix with an acidic pH, a dry plant matrix and a fatty plant matrix.

The method of analysis according to the invention can be used for the analysis of a sample of
plants chosen from wheat, barley, potato, cotton, proteinaceous crops, oil-bearing crops, maize,
flax, rice, vegetable crops, fruit trees, grapevine and beetroot.

For the method for analyzing a plant tissue sample according to the invention, the preparation step may be an extraction of the plant tissues. This preparation step may also comprise a concentration of the sample.

5

For the method for analyzing a plant tissue sample according to the invention, the dilution step can be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid, acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be acidified ; or alternatively in a mixture of such solvents.

10

According to a third particular aspect of the invention, it relates to a method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 5 mg/kg, preferably less than or equal to 0.05 mg/kg, more particularly less than or equal to 0.005 mg/kg of a soil sample, comprising the following steps:

15

- preparation of the soil sample ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

20

The method for analyzing a soil sample according to the invention may comprise a step consisting of dilution of the sample prepared.

25

The method for analyzing a soil sample according to the invention is suitable for the simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

30

Preferably, the method for analyzing a soil sample according to the invention is used for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ; phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or one or more salts thereof ; more preferably for the analysis of fosetyl-Al.

Particularly advantageously, the method for analyzing a soil sample according to the invention is used for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

35

The method for analyzing a soil sample according to the invention can be used for the analysis of any type of soil, for example clayey, sandy or chalky soil.

The method for analyzing soil according to the invention can be used for cultivated soils or for bare soils, in particular before a crop or after harvest.

For the method for analyzing a soil sample according to the invention, the preparation step may be an extraction of the soil sample. This preparation step may also comprise a concentration of the sample.

5

For the method for analyzing a soil sample according to the invention, the dilution step can be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid, acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be acidified ; or alternatively in a mixture of such solvents.

10

According to a fourth particular aspect of the invention, it relates to a method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.1 mg/m^3 , preferably less than or equal to 0.01 mg/m^3 , more particularly less than or equal to 0.001 mg/m^3 of an air sample, comprising the following steps:

15

- preparation of the air sample ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

20

The method for analyzing an air sample according to the invention may comprise a step consisting of dilution of the sample prepared.

25

The method for analyzing an air sample according to the invention is suitable for the simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

30

Preferably, the method for analyzing an air sample according to the invention is used for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ; phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or of one or more salts thereof ; more preferably for the analysis of fosetyl-Al.

35

For the method for analyzing an air sample according to the invention, the preparation step may be a trapping.

For the method for analyzing an air sample according to the invention, the dilution step can be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid,

acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be acidified ; or alternatively in a mixture of such solvents.

According to a fifth particular aspect of the invention, it relates to a method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.00005 mg/kg, preferably less than or equal to 0.000005 mg/kg, more preferably less than or equal to 0.0000005 mg/kg of a sample of a human body fluid, comprising the following steps:

- preparation of the human body fluid sample ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

The method for analyzing a sample of a human body fluid according to the invention may comprise a step consisting of dilution of the sample prepared.

The method for analyzing a sample of a human body fluid according to the invention is suitable for the simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

Preferably, the method for analyzing a sample of a human body fluid according to the invention is used for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ; phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or of one or more salts thereof ; more preferably for the analysis of fosetyl-Al.

Particularly advantageously, the method for analyzing a sample of a human body fluid according to the invention is used for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

Preferably, the method for analyzing a sample of a human body fluid according to the invention can be used for the analysis of a sample chosen from human blood and human urine.

For the method for analyzing a sample of a human body fluid according to the invention, the dilution step can be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid, acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be acidified ; or alternatively in a mixture of such solvents.

According to a sixth particular aspect of the invention, it relates to a method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 1 mg/kg, preferably less than or equal to 0.01 mg/kg, more preferably less than or equal to 0.001 mg/kg of a sample of animal products or tissues, comprising the following steps:

- preparation of the sample of animal products or tissues ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

5

The method for analyzing a sample of animal products or tissues according to the invention may comprise a step consisting of dilution of the sample prepared.

10

The method for analyzing a sample of animal products or tissues according to the invention is suitable for the simultaneous analysis of several pesticidal compounds which may be fungicidal, herbicidal, insecticidal or growth-regulating compounds.

15

Preferably, the method for analyzing a sample of animal products or tissues according to the invention is used for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ; phosphonic acid or a derivative thereof ; preferably for the analysis of fosetyl or of one or more salts thereof ; more preferably for the analysis of fosetyl-Al.

20

Particularly advantageously, the method for analyzing a sample of animal products or tissues according to the invention is used for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

25

Preferably, the method for analyzing a sample of animal products or tissues according to the invention can be used for the analysis of a sample chosen from milk, eggs, liver, kidneys, fats and muscle.

30

For the method for analyzing a sample of animal products or tissues according to the invention the dilution step can be carried out in an aqueous solvent, which may be acidified, preferably chosen from formic acid, acetic acid or trifluoroacetic acid ; or in an organic solvent, preferably acetonitrile or methanol, which may be acidified ; or alternatively in a mixture of such solvents.

35

For the method for analyzing a sample of animal products or tissues according to the invention, the preparation step may be an extraction of the animal products or tissues. This preparation step may also comprise a concentration of the sample.

According to a seventh particular aspect of the invention, it relates to a method for analyzing one or more pesticidal compounds present in a converted agrofood product sample. This method for analyzing a converted agrofood product sample according to the invention is similar to the method for analyzing a plant product sample according to the invention, in which the plant product sample is replaced with a converted agrofood product sample.

The various steps and preferences are also similar.

For the various aspects of the invention, in the analysis step, the external standards used are prepared in the presence of a matrix of the same nature as the sample to be analyzed.

The examples which follow are given by way of illustration of the various aspects of the invention. These examples do not limit the scope of the invention. In particular, those skilled in the art will be able to adapt or modify some of the steps of the method of analysis according to the invention according to the specific needs with which they will be confronted. Such modifications or adaptations are part of the scope of the present invention.

Example 1

15

This example concerns the analysis of fosetyl-Al and of phosphorous acid using plant tissue samples. The plant matrices are derived from crops: of cucumber, of orange, of lettuce, of grape and of avocado.

20 Procedure for using the method of analysis according to the invention:

1. Weigh 20.0 g of homogenized plant matrix sample into a 125 ml polypropylene flask.
2. Add 80 ml of the water/acetonitrile mixture (50/50, volume/volume).
3. Mill the sample for 5 minutes using an IKA T25-type mill.
- 25 4. Centrifuge for 5 minutes at 3600 rpm at 5°C.
5. Transfer the supernatant into a 200 ml volumetric flask.
6. Take up the pellet with 80 ml of the water/acetonitrile mixture (50/50, volume/volume).
7. Mill the sample again for 5 minutes.
- 30 8. Centrifuge for 5 minutes at 3600 rpm at 5°C.
9. Transfer the supernatant into the 200 ml volumetric flask.
10. Adjust to 200 ml using methanol.
11. Centrifuge an aliquot of approximately 10 ml for 10 minutes at 6000 rpm at ambient temperature.
- 35 12. Filter the supernatant through a PTFE filter (of the type Acrodisc CR 25 mm, 0.45 µm).
13. Dilute the filtrate 5 times using methanol acidified with 0.5% formic acid.
14. Analyze by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS), or LC/MS/MS analysis.

To implement the analysis, the calibration is carried out by external standardization. The standards used must be prepared in a matrix of the same nature as the samples that are the subject of the specific analysis.

5

Analytical conditions for step 14:

High performance liquid chromatography (HPLC) conditions:

10 Column: Hypercarb, 100 × 3.0 mm, 5 µm
Precolumn: Phenomenex C18 ODS, 4 × 2.0 mm
Mobile phase: water acidified with 0.5% formic acid/methanol (65/35, volume/volume) isocratic mode
15 Flow rate: 400 µl/min
Column temperature: ambient
Injection volume: 20 µl

Before the analysis, the chromatographic system is left to stabilize for approximately 2 hours.

20

Tandem mass spectrometry (MS/MS) conditions:

Detector: triple quadrupole, type API4000 Sciex Instrument
Interface: TIS (Turbo Ion Spray)
Scan type: MRM (Multiple Reaction Monitoring) mode
25 Polarity: negative
Gas inlet pressure: nitrogen: 4 bar
air: 7.5 bar
air (exhaust): 4 bar
Gas flow rate: nebulizing gas (air, GS1): 40
30 turbo gas (air, GS2): 60
protective gas (nitrogen, CUR): 20
collision gas (nitrogen, CAD): 6
High voltage TIS (IS): -4500 V
Source temperature: 500°C
35 Entry potential (EP): -10 V

Collision energy:

Compound	Precursor ion Q1 mass (amu)	Product ion Q3 mass (amu)	Dwell time (msec)	Collision energy (CE) (eV)	Decluster- ing potential (V)	Collision cell exit potential (CXP) (V)
fosetyl-Al	109.0	80.9	200	-16	-45	-1
	109.0	63.0	200	-38	-45	-5
H ₃ PO ₃	80.9	63.0	600	-38	-55	-1
	80.9	78.9	600	-22	-55	-5

When the method of analysis according to the invention is used, steps 1 to 12 concern the preparation of the sample, and step 13 concerns the dilution. Thus, step 14 concerns the LC/MS/MS analysis of the plant matrix sample prepared beforehand and then diluted.

The results obtained from the analysis of various plant matrices are given in detail in the tables below, in which the CV values indicate the coefficient of variation. According to the present invention, the CV values can also correspond to RSD values.

These results were obtained from control samples which were enriched in fosetyl-Al and in phosphorous acid to limits of quantification (0.1 mg/kg for phosphorous acid and 0.01 mg/kg for fosetyl-Al) and to 10 times these limits.

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.100	83	82	82	91	95	87	7.0
1.000	102	102	101	97	99	100	2.2
	Total CV [%]					93	8.9

Cucumber - phosphorous acid

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.010	95	96	98	98	95	96	1.6
0.100	106	101	101	99	100	101	2.7
	Total CV [%]					99	3.4

Cucumber - fosetyl-Al

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.100	65	99	70	78	67	76	18.3
1.000	97	99	101	95	99	98	2.3
	Total CV [%]					87	17.3

Orange - phosphorous acid

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.010	102	100	98	87	97	97	6.0
0.100	96	95	94	90	86	92	4.5
	Total CV [%]					95	5.6

5

Orange - fosetyl-AI

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.100	82	94	76	79	74	81	9.7
1.000	103	108	98	106	107	104	3.9
	Total CV [%]					93	14.7

Lettuce - phosphorous acid

10

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.010	104	98	109	113	114	108	6.2
0.100	102	106	104	107	107	105	2.1
	Total CV [%]					106	4.5

Lettuce - fosetyl-AI

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.100	96	94	113	100	92	99	8.5
1.000	100	105	104	101	101	102	2.1
	Total CV [%]					101	6.0

Grape - phosphorous acid

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.010	102	102	95	101	100	100	2.9
0.100	101	102	102	102	100	101	0.9
	Total CV [%]					101	2.1

Grape - fosetyl-Al

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.100	74	77	68	70	75	73	5.1
1.000	110	97	92	103	100	100	6.7
	Total CV [%]					87	17.8

5

Avocado - phosphorous acid

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.010	104	95	92	94	93	96	5.0
0.100	88	88	85	89	86	87	1.9
	Total CV [%]					91	6.1

Avocado - fosetyl-Al

Example 2:

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This example also concerns the analysis of fosetyl-Al and of phosphorus acid using plant tissue samples. The plant matrices are derived from wheat crops.

This example repeats the conditions of Example 1 up to step 9, and then steps 10 to 14 of Example 1 are replaced with the following steps:

10. Add 1 ml of pure formic acid and adjust to 200 ml using methanol.
 11. Centrifuge an aliquot of approximately 10 ml for 10 minutes at 6000 rpm at ambient temperature.
 12. Dilute the supernatant twice using methanol acidified with 0.5% formic acid.
 13. Filter through a PTFE filter (type Acrodisc CR 25 mm, 0.45 µm).
 14. Analyze by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS), or LC/MS/MS analysis.
- For the rest, this example is identical to Example 1.

The results obtained from the analysis of a wheat sample are given in detail in the table below, in which CV indicates the coefficient of variation.

- These results were obtained from control samples which were enriched in fosetyl-Al and in phosphorous acid to the limits of quantification (0.1 mg/kg for phosphorous acid and 0.01 mg/kg for fosetyl-Al) and to 10 times these limits.

Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.100	90	94	101	102	103	98	5.8
1.000	77	77	81	79	81	79	2.5
Total CV [%]						89	12.2

Wheat - phosphorous acid

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Quantification level mg/kg	Recovery range [%]					Means [%]	CV [%]
0.010	86	92	93	85	82	88	5.4
0.100	75	79	69	71	71	73	5.5
Total CV [%]						80	10.9

Wheat - fosetyl-Al

For these two examples, the results obtained are in accordance with the regulatory provisions (96/48/EC of 16 July 1996).

25

Furthermore, these results made it possible to attain limits of quantification that are below the limits available with the previously known methods.

These examples also demonstrate the simplicity and the greater safety of the method according to the invention.

Example 3

This detailed example concerns the analysis of fosetyl-Al and of phosphorous acid using plant tissue samples. This example is a modification M001 to the Analytical Method 00861 for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in/on grape, orange, lettuce, cucumber, avocado and wheat by LCMSMS.

Description:

15 Data Requirement:

EU Council Directive 91/414/EEC amended by Commission Directive 96/68/EC

European Commission Guidance Document for Generating and Reporting Methods of Analysis in Support of P...Registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414, SANCO/3029/99

20 European Commission Guidance Document for on Residue Analytical Methods, SANCO/825/00

Summary:

The presented residue analytical method modification 00861/M001 was validated for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in grape (whole fruit), orange (whole fruit), lettuce (head), cucumber (whole fruit), avocado (whole fruit), and wheat (grain) by LC/MS/MS.

fosetyl-Al and its metabolite (phosphorous acid) were extracted from the sample material with a mixture of acetonitrile/water (50/50). After centrifugation and dilution of the sample material, the residues are quantified by HPLC using an Hypercarb column and detected by tandem mass spectrometry with electrospray ionisation. The quantification was done by an external standardisation in matrix matched standards

The validation set included the determination of the detector linearity, the limit of quantification, the accuracy of the method and the storage stability of sample final extracts.

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at respective concentrations between 0.1 and 5 µg/L and 1 and 50 µg/L, except for wheat samples between 0.31 and 8.3 µg/L and 3.1 and 83 µg/L.

The occurrence of matrix effects was monitored. In all the sample materials, the measurement of phosphorous acid must be established using matrix matched standards. So the measurement of both compounds is established using matrix matched standards.

The apparent residues for all control samples were below 30% of the LOQ for each compound, i.e. < 0.003 mg/kg of fosetyl-Al and 0.03 mg/kg of phosphorous acid.

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq 20\%$ could be obtained. The LOQ was set at 0.01 mg/kg for fosetyl-Al and at 0.1 mg/kg for phosphorous acid in grape (whole fruit), orange (whole fruit), lettuce (head), cucumber (whole fruit), avocado (whole fruit), and wheat (grain).

The accuracy of the method can be assessed on the basis of the determined recovery rates. The single recovery rates were in the range of 69 to 114 % for fosetyl-Al and of 65 to 113 % for phosphorous acid. The mean recovery rates per fortification level were in the range of 93 to 97 % for fosetyl-Al with an overall recovery rate over all sample materials and fortification levels of 95% and of 86 to 97 % for phosphorous acid with an overall recovery rate over all sample materials and fortification levels of 91 %. The accuracy of the method fulfils the requirements for residue analytical methods which demand that the mean recoveries for each fortification level should be in the range of 70-110%.

The precision and repeatability of the method can be assessed on the basis of the determined relative standard deviations (RSD) for the mean values of the recovery rates. The relative standard deviations (RSD) for the single fortification levels ranged from 7.6 to 12.3 % for fosetyl-Al and from 9.5 to 14.9 % for phosphorous acid (n=30).

The relative standard deviations (RSD) for the single fortification levels ranged from 7.6 to 12.3 % for fosetyl-Al and from 9.5 to 14.9 % for phosphorous acid (n=30). The overall RSD values per sample material were between 2.1 and 10.9 % for fosetyl-Al and 6.0 and 17.8 % for phosphorous acid (n=10). The RSD value across all samples was 10.2 % for fosetyl-Al and 13.7 % for phosphorous acid (n=60).

The overall RSD values per sample material were between 2.1 and 10.9 % for fosetyl-Al and 6.0 and 17.8 % for phosphorous acid (n=10). The RSD value across all samples was 10.2 % for fosetyl-Al and 13.7 % for phosphorous acid (n=60). All RSD values were well below 20%, so that the precision and repeatability of the method can be considered acceptable.

All results of the method validation are in accordance with the general requirements for residue analytical methods, so that this method modification has been validated successfully.

1 Introduction

Fosetyl-Al is a fungicide

The method modification 00861/M001 presented in this report was validated in order to suppress the steps of clean up and derivatization, to change the analysis and detection modes

and to decrease the Limit of Quantification (LOQ) of original method 00861 from 0.5 mg/kg to 0.01 mg/kg for fosetyl-Al and to 0.1 mg/kg for phosphorous acid.

Table 1: LOQ and principle of analytical determination

Compound		fosetyl-Al	Phosphorous acid
Determined as		fosetyl-Al	Phosphorous acid
Calculated as		fosetyl-Al	Phosphorous acid
Principle of Determination		LC/MS/MS	LC/MS/MS
LOQ [mg/kg]	Grape (whole fruit)	0.01	0.1
	Orange (whole fruit)	0.01	0.1
	Lettuce (head)	0.01	0.1
	Cucumber (whole fruit)	0.01	0.1
	Avocado (whole fruit)	0.01	0.1
	Wheat (Grain)	0.01	0.1

5 1. defined as the lowest validated fortification level

1.1 Citation of the Original Method

Original Method: 00861

Compounds: fosetyl-Al and its metabolite (phosphorous acid)

Reason for Modification:

- Suppress the steps of clean up and derivatization
- Change analysis and detection modes from GC/FPD to LC/MS/MS
- Decrease the LOQ from 0.5 mg/kg to 0.010 mg/kg for fosetyl-Al and to 0.1 mg/kg for phosphorous acid

1.2 Physical and Chemical Properties

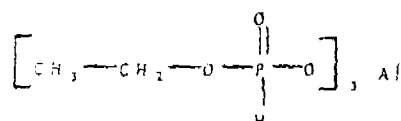
Name of the Substance fosetyl-Al

Substance Code AE F053616

Chemical Name Aluminium-tris-(ethylphosphonate)

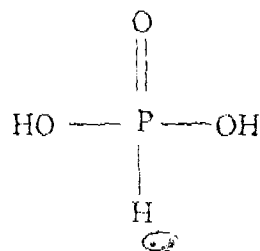
Empirical Formula $C_6H_{15}AlO_9P_3$

Structural Formula



Relative Molecular Mass	354.1 g/mol
Monoisotopic Mass	354.0 g/mol
Solubility	Water 120 g/L (20°C) Acetonitrile 5 mg/L (20°C)
Name of the Substance	Phosphorous acid
Substance Code	AE 0540099
Chemical Name	Phosphonic acid
Empirical Formula	H ₃ PO ₃

Structural Formula



Relative Molecular Mass	82.0 g/mol
Monoisotopic Mass	82.0 g/mol

2 Experimental Section

2.1 Materials

2.1.1 Apparatus

- Standard laboratory glass equipment, rinsed with acetone.
- 5 • Balances :
 - accuracy ± 0.1 mg (analytical standards) (e.g. Mettler AT261 range)
 - accuracy ± 0.1 g (samples) (e.g. Mettler PM6000)
- Dilutor (e.g. Hamilton MicroLab 500)
- 10 • High-speed blender (e.g. Ultra Turrax T25 with dispersion tool S 50 G-40G) (e.g. IKA)
- Centrifuge (e.g. Hermle Z513K)

- HPLC (ex Hettich EBA12)
(e.g. Binary Pump Agilent 1100)
(e.g. Quaternary pump Agilent 1100)
- Auto sampler (e.g. CTC Analytics HTC PAL)
- Triple Quadrupole HPLC-MS/MS Mass Spectrometer (e.g. Sciex Instruments, API 4000 System)
- Column (e.g. Hypercarb, 100 x 3.0 mm, 5 µm)

2.1.2 Reagents and Supplies

- Acetone, (e.g. SupraSolv® Merck)
- Acetonitrile, (e.g. SupraSolv® Merck)
- Methanol (e.g. SupraSolv® Merck)
- Formic acid (e.g. Normapur Prolabo)
- PTFE filters (25mm, 0.45 µm), (e.g. Acrodisc CR Pall Gelman)
- Polypro bottles (125 mL, wide opening) (e.g. Nalgen)
- Conical centrifuge tube 15 mL (e.g. Merck 01 142.518)
- Extraction solvent : Acetonitrile / water (50/50, v/v)
- Solvent for dilution Methanol with 0.5 % HCOOH
- Mobile phase solvent: Water with 0.5 % HCOOH

2.1.3 Reference Item

Only sufficiently characterised and certified item was used as reference item.
The reference item was made available by Bayer CropScience GmbH produkt Analytik, G864, Industriepark Höchst, D-65926 Frankfurt-am-Main, Germany.

Table 2: Reference item data

Name of Substance	Batch Number	Content [%]	Date of Expiry
fosetyl-Al	12/1080	97.6	February 20, 2006
phosphorous acid	04911DN	96.2	March 12, 2005

2.1.4 Standard Solutions

Stock and standard solutions were stored protected from light in a refrigerator at around 5°C.

Stock solutions (1000 mg/L)

- Into a 100 mL amber screw-cap flask, weigh accurately between 20 and 50 mg of reference item. Using a burette, add a volume of water to obtain a stock solution of exactly 1000 mg/L. Mix thoroughly until complete dissolution using a magnetic stirrer. Two separate stock solutions

must be prepared for each compound. After comparison of these two stock solutions, they are mixed.

Mixture solution

First dilute 10 times fosetyl-AI stock solution with water. Then pipette 5 mL of it and 5 mL of phosphorous acid stock solution using a class "A*" pipette. Pour into a class "A*" 50 mL volumetric flask. Adjust volume with water, cap and mix by shaking : Mixture solution (10 mg/L fosetyl-AI - 100 mg/L phosphorous acid).

Fortifying standard solutions

The mixture solution is also used as fortifying standard solution for recoveries at 10LOQ level. Dilute it 10 times with water to obtain the fortifying standard solution used for recoveries at LOQ level.

Standard solutions in solvent

By appropriate dilution of the fortifying solution used for recoveries at LOQ level (1 mg/L fosetyl-AI - 10 mg/L phosphorous acid), prepare the intermediate standard solution at 0.05 mg/L of fosetyl-AI and 0.5 mg/L of phosphorous acid using methanol with 0.5% formic acid.

Standard solutions in solvent

To obtain the standard solutions used for calibration, dilute extemporaneously using a dilutor and methanol with 0.5% formic acid, the intermediate standard solution to obtain the following concentrations: 0.1 µg/L fosetyl-AI - 1 µg/L phosphorous acid, 0.2 µg/L fosetyl-AI - 2 µg/L phosphorous acid, 0.5 µg/L fosetyl-AI - 5 µg/L phosphorous acid, 1 µg/L fosetyl-AI - 10 µg/L phosphorous acid, 2 µg/L fosetyl-AI - 20 µg/L phosphorous acid and 5 µg/L fosetyl-AI - 50 µg/L phosphorous acid.

For wheat samples only, dilute extemporaneously using a dilutor and methanol with 0.5% formic acid, the intermediate standard solution to obtain the following concentrations : 0.31 µg/L fosetyl-AI - 3.1 µg/L phosphorous acid, 0.5 µg/L fosetyl-AI - 5 µg/L phosphorous acid, 0.83 µg/L fosetyl-AI - 8.3 µg/L phosphorous acid, 1 µg/L fosetyl-AI - 10 µg/L phosphorous acid, 2.5 µg/L fosetyl-AI - 25 µg/L phosphorous acid, 5 µg/L fosetyl-AI - 50 µg/L phosphorous acid and 8.3 µg/L fosetyl-AI - 83 µg/L phosphorous acid.

Matrix matched standard solutions used for calibration

The occurrence of matrix effects was monitored, and the measurement of both compounds is established using matrix matched standards in all the sample materials.

From the intermediate standard solution, the dilutions are the same as preparation of standard solutions in solvent, except the dilution mixture which is the final extract of a control sample.

Remark : 20 to 25 mL of final extract are necessary to make all dilutions. If the step of filtration is difficult, several filters can be used.

2.1.5 Stability of the standard solutions

The stock solutions, stored protected from light in a refrigerator at around 5°C, were found to be stable for 4 months and a half.

The fortifying standard solutions, stored protected from light in a refrigerator at around 5°C, were found to be stable for 2 months and a half.

2.2 Residue Analytical Methodology

Some modifications compared to the original analytical method were introduced :

The method modification 00861/M001 presented in this report was validated in order to suppress the steps of clean up and derivatization, to change the analysis and detection modes and to decrease the Limit of Quantification (LOQ) of original method 00861 from 0.5 mg/kg to 0.01 mg/kg for fosetyl-Al and to 0.1 mg/kg for phosphorous acid.

- The Limit of Quantification was decreased from 0.5 mg/kg to 0.01 mg/kg for fosetyl-Al and to 0.1 mg/kg for phosphorous acid.
- The C18 SPE cartridge clean-up step was suppressed.
- The derivatization step was suppressed.
- The quantification was carried out by LC/MS/MS instead of GC/FPD.

All modifications were included in the description below.

A flow chart of the method is given in Appendix 1.

For recovery experiments, samples are fortified by adding the appropriate standard solution to the sample material after weighing and before extraction.

Extraction

1. Weigh 20.0 g of homogeneous sample material into a 125 mL polypropylene bottle.
Note: weight of the sample is used for residue calculation, addressed as variable G
2. Add 80 mL of acetonitrile / water (50/50, v/v).

3. Blend the sample using a high-speed blender (IKA or equivalent) for approx. 5 minutes.
4. Centrifuge the extract (3600 rpm – 5°C) for approx. 5 minutes.
5. Pour the supernatant into a 200 mL volumetric flask.
6. Add 80 mL of acetonitrile / water (50/50, v/v) on the bottom.
7. Blend the sample using a high-speed blender (IKA or equivalent) for approx. 5 minutes.
8. Centrifuge the extract (3600 rpm – 5°C) for approx. 5 minutes.
9. Pour the supernatant into the volumetric flask.
10. Make-up to 200 mL with methanol. This is the Extract A.
Note: volume of extract A is used for residue calculation, addressed as variable V
11. Centrifuge an aliquot of about 10 mL of Extract A (6000 rpm – ambient) for approx. 10 minutes.
12. Filter the supernatant through an Acrodisc CR 25 mm PTFE filter (0.45 µm).
13. Dilute five times the extract using acidified methanol with formic acid 0.5 %. This is the Final Extract.
14. Proceed to LC/MS/MS measurement, Chapter 2.3.

Remark : for wheat samples, from stage 10, follow the preparation as described below :

10. Add 1 mL of concentrated formic acid and make-up to 200 mL with methanol. This is the Extract A.
Note: volume of extract A is used for residue calculation, addressed as variable V
11. Centrifuge an aliquot of about 10 mL of Extract A (6000 rpm – ambient) for approx. 10 minutes.
12. With dilutor, dilute twice the supernatant using methanol with formic acid 0.5 %
13. Filter the extract through an Acrodisc CR 25 mm PTFE filter (0.45 µm). This is the Final Extract.
14. Proceed to LC/MS/MS measurement, Chapter 2.3.

2.3 Analysis and Instrument Conditions

The final extracts are injected into a high performance liquid chromatograph and detected by tandem mass spectrometry with electrospray ionisation.

The quantification is carried out by external standardisation using matrix matched standards.

Exemplary LC/MS/MS conditions that were used in the course of this method validation are listed in chapters 2.3.1 and 2.3.2. These conditions are given as a guidance and may have to be adapted for other HPLC-MS/MS systems.

2.3.1 HPLC Conditions

Instrument:	Binary pump Agilent 1100
	Quaternary pump Agilent 1100 (make-up solvent)
Auto sampler:	CTC Analytics HTC PAL

- Column: Hypercarb, 100 x 3.0 mm, 5 µm
- Precolumn: Phenomenex C13 ODS, 4 x 2.0mm
- Injection Volume: 20 µL
- Column temperature: ambient (about 25°C)
- 8 Mobile Phase: Isocratic mode: 35 / 65 (v/v) Methanol / water + 0.5% formic acid
- Flow (Column): 400 µL/min
- Retention Times: from 4 to 5 min for phosphorous acid and from 7 to 10 min for fosetyl-Al.
- 12 Divert valve. Valve situated between the analytical column and the MS/MS system.
- This valve was used to protect the ion source from contamination and to reduce the risk of ion suppression occurring, the eluent from the first minutes of the run was diverted to waste and a make-up flow was used to obviate the need for the ion source to stabilize after diverting the LC eluent flow back to the mass spectrometer.
- 16 Make up solvent: 50 / 50 (v/v) Methanol / water
- Divert flow: 200 µL/min
- Remarks :
- It is necessary to wait about 2 hours the stabilisation of the HPLC system before injecting.
 - During a samples set, a light drift of retention time of both compounds can be observed.
 - Hypercarb precolumn must not be used.
- 20

2.3.2 MS/MS Conditions

- The experiments were performed on a triple-quadrupole mass spectrometer system, fitted with
- 25 an electrospray interface operated in the negative ion mode under MRM (multiple reaction monitoring) conditions.

For instance:

- Detector: Triple Quadrupole HPLC-MS/MS Mass Spectrometer,
- 30 e.g. Sciex Instruments, API 4000 System
- Source: TIS (Turbo Ion Spray)
- Temperature: 600°C
- Scan Type: MRM-Mode (Multiple Reaction Monitoring Mode)

Polarity: Negative ion mode

Gas Flows: Nebulization Gas Air (GS1):40

Turbo Gas Air (GS2): 60

Curtain Gas N₂ (CUR): 20

Collision Gas N₂ (CAD): 6Collision Energy:

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (V)
fosetyl-Al	109.0	80.9	200	-38
Phosphorous acid	80.9	62.9	600	-16

Table 3 Mass spectrometer scan parameters for the quantifier ions used. The detailed instrument settings used are given in chapters 2.3.1 and 2.3.2. Varying instrument systems or instrument parameters may result in different ion transitions and different relative intensities.

Note: Some mass spectrometer conditions are instrument specific. The spectrometer conditions should be optimised by a competent operator prior to analysis.

Details on MS/MS and LC conditions are given in Appendix 2.

The fragmentation pathways for the quantifier ions for fosetyl-Al and phosphorous acid are shown in Figure 1 and in Figure 2.

Figure 1: Proposed fragmentation pathway for fosetyl-Al.

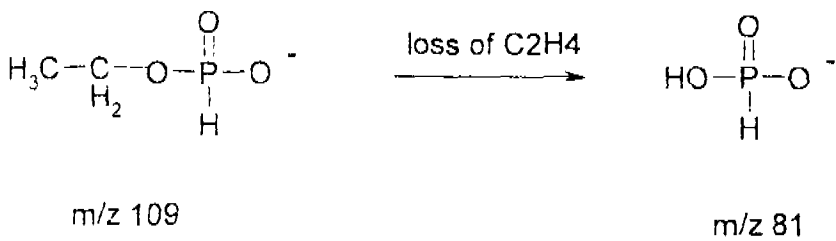
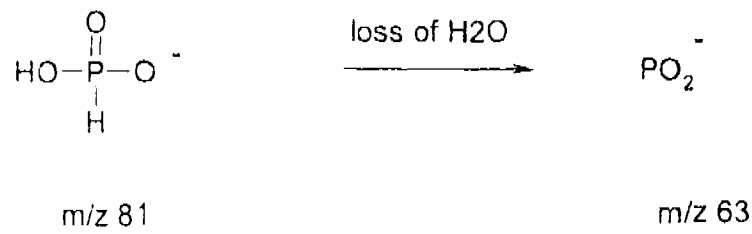


Figure 2 Proposed fragmentation pathway for phosphorous acid.



2.3.3 Confirmatory transitions

- To confirm or exclude some interference or pollution in samples, the following transitions can be used in the same conditions described above :

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (V)
fosetyl-Al	109	63	200	-38
phosphorous acid	80.9	78.9	600	-22

The fragmentation pathways for the confirmatory transitions for fosetyl-Al and phosphorous acid are shown in Figure 3 and in Figure 4.

Figure 3: Proposed fragmentation pathway for fosetyl-Al

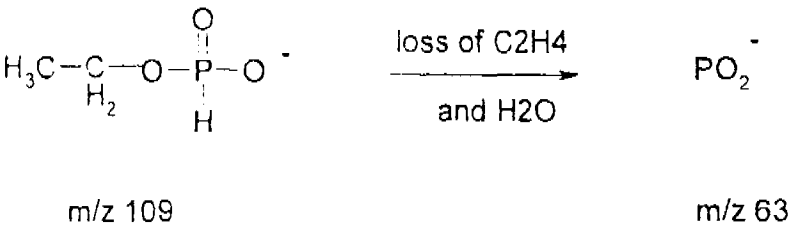
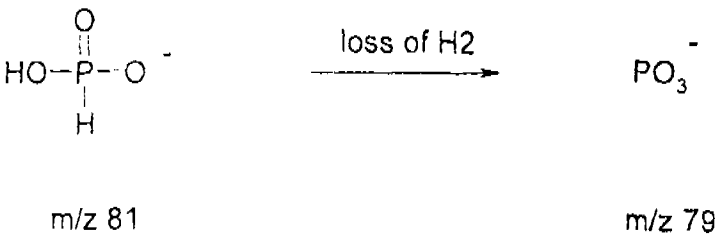


Figure 4. Proposed fragmentation pathway for phosphorous acid



Note : all recovery samples were also analysed using confirmatory transitions. The results are given in Appendix 3.

5 2.4 Linearity of the Detector

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at respective concentrations between 0.1 and 5 µg/L and 1 and 50 µg/L, except for wheat samples between 10 0.31 and 8.3 µg/L and 3.1 and 83 µg/L.

Table 4 : Standard concentrations prepared for the determination of detector linearity. The concentration corresponding to the LOQ is given in the second column of the table.

HPLC-MS/MS	Standard Concentrations [µg/L]					
fosetyl-Al	0.1	0.2	0.5	1	2	5
phosphorous acid	1	2	5	10	20	50

15 Table 5: Standard concentrations used for wheat samples and prepared for the determination of detector linearity. The concentration corresponding to the LOQ is given in the second column of the table.

HPLC-MS/MS	Standard Concentrations [µg/L]						
fosetyl-Al	0.31	0.5	0.83	1	2.5	5	8.3
phosphorous acid	3.1	5	8.3	10	25	50	83

2.5 Storage Stability of Extracts

The stability of sample extracts containing fosetyl-Al and phosphorous acid was determined for each sample material.

For this purpose, the final extract of recovery samples were stored in the auto sampler rack thermo stated at about 10 °C after initial analysis and re-analysed after a storage period of several days.

2.6 Calculation

2.6.1 Calculation of Residues

Evaluation in this case is performed according to the external standardisation using matrix matched standards.

During the analysis of each set of samples, the 6 (or 7 for wheat samples) standard solutions mentioned in Table 4 are injected once. Standards should be interspersed with samples to compensate for any minor change in instrument response.

For each compound, the peak area is plotted versus the concentration in order to establish a calibration curve obtained by linear regression weighting 1/x with least squares method.

The corresponding model to determine the concentration in final extracts is calculated using the Analyst Software (Version 1.4).

Each final extract is injected once using the same conditions as previously described for the standard solutions.

Using the predicting mathematical model previously established, the final concentration in µg/L of each compound is determined for each injection.

For each compound, the amount of residue R, expressed in mg/kg is calculated, using the following formula :

$$R = \frac{C \times V \times D}{1000 \times G}$$

- where:
- R : Determined amount of residue of fosetyl-Al or phosphorous acid in mg/kg
 - C : Concentration of fosetyl-Al or phosphorous acid found in the analysed extract in µg/L
 - V : Volume of the extract A in mL, here 200 mL
 - D : Dilution factor to obtain the Final Extract, here 5 (or 2 for wheat samples)
 - G : Sample weight of analytical sample in g, here 20 g

2.6.2 Calculation of Recovery Rates

The concentration of each compound in µg/L is determined for the recovery sample according to 2.6.1.

The percent recovery rate is then calculated as follows :

5

$$Rec = \frac{C \times 100}{A}$$

where: Rec : Recovered amount found in fortified sample in %

C : Concentration of fosetylAl or phosphorous acid found in the analysed extract in µg/L

A : Fortified amount of fosetyl-Al or phosphorous acid in µg/L

10 2.6.3 Calculation of Relative Standard Deviation (RSD)

The RSD is calculated as follows :

$$RSD (\%) = S.D. / \text{Mean Recovery} \times 100 \%$$

$$S.D. = \left[\frac{\sum (R_i - R_m)^2}{n - 1} \right]^{1/2}$$

R_i : recovery

R_m : mean recovery

n : number of recoveries

3 Results and Discussion

15 3.1 Specificity and Selectivity

The method allows the determination of fosetyl-Al and its metabolite (phosphorous acid) in/on grape, orange, lettuce, cucumber, avocado and wheat samples.

The specificity of the method resulted from the HPLC separation in combination with the very selective MS/MS detection.

20

3.2 Apparent residues in Control Samples

Two control samples were analysed for each sample material. The origin of the control materials used is listed in Table 6.

Table 6: Origin of untreated control samples

Sample Material	Origin
Grape (whole fruit)	Mr Lusson – Angers - France
Orange (whole fruit)	Market specialised in organic food - France
Lettuce (head)	Market specialised in organic food - France
Cucumber (whole fruit)	Market specialised in organic food - France
Avocado (whole fruit)	Control sample from Bayer CropScience – Monheim - Germany
Wheat (Grain)	Control sample from Bayer CropScience – Monheim - Germany

A residue level estimation in control samples was performed. The results are listed in Table 7. The apparent residues for all control samples were below 30% of the LOQ for each compound, i.e. < 0.003 mg/kg of fosetyl-Al and < 0.03 mg/kg of phosphorous acid.

s Table 7: Apparent residues in untreated control samples for fosetyl-Al and phosphorous acid

Sample Material	Control Sample	LOQ [mg/kg]		Apparent residues [mg/kg]	
		fosetyl-Al	phosphorous acid	fosetyl-Al	phosphorous acid
Grape (whole fruit)	Mr Lusson – Angers - FRANCE	0.01	0.1	< 0.003	< 0.03
Orange (whole fruit)	Market specialised in organic food - France	0.01	0.1	< 0.003	< 0.03
Lettuce (head)	Market specialised in organic food - France	0.01	0.1	< 0.003	< 0.03
Cucumber (whole fruit)	Market specialised in organic food - France	0.01	0.1	< 0.003	< 0.03

Avocado (whole fruit)	Control sample from Bayer CropScience – Monheim - Germany	0.01	0.1	< 0.003	< 0.03
Wheat (Grain)	Control sample from Bayer CropScience – Monheim - Germany	0.01	0.1	< 0.003	< 0.03

3.3 Linearity of the Detectors and Matrix Effects

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

- 5 The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at respective concentrations between 0.1 and 5 µg/L and 1 and 50 µg/L, except for wheat samples between 0.31 and 8.3 µg/L and 3.1 and 83 µg/L.

Experimental details can be found in Chapter 2.4.

- 10 In each chromatogram, the measured peak area of fosetyl-Al or phosphorous acid is plotted versus the corresponding concentration of respectively fosetyl-Al or phosphorous acid contained in each standard solution, in order to obtain calibration curve of the form :

$$y = ax + b \quad (1/x \text{ weighting})$$

where . y = peak area,
 x = concentration in injected standard solution

- 15 The results of the determination of detector response for LC/MS/MS are summarised in Table 8.

Table 8 : Summary of the determination of detector linearity for LC/MS/MS.

Detection	Parameter	fosetyl-Al	phosphorous acid
LC/MS/MS	Linear range [µg/L]	0.1 – 5 (or 0.31 – 8.3 for wheat)	1 – 50 (or 3.1 – 83 for wheat)
	No. of concentrations	6 (or 7 for wheat)	6 (or 7 for wheat)

No. of injections	1	1
Model	1/x weighted linear regression	1/x weighted linear regression
Correlation coefficient (R) for standards prepared in solvent	> 0.9990	> 0.9900
Correlation coefficient (R) for matrix matched standards	> 0.9990	> 0.9985

An excellent linear correlation between the injected amount of the analytes and the detector responses of LC/MS/MS was observed for standards in the range of 0.1 to 5 µg/L (or 0.31 to 8.3 µg/L for wheat) for fosetyl-Al and in the range of 1 to 50 µg/L (or 3.1 to 83 µg/L for wheat) for phosphorous acid, using either standards prepared in solvent or matrix matched standards.

The occurrence of matrix effects was monitored. The results are shown in Table 9 and table 10.

Table 9: Matrix effect evaluation for fosetyl-Al
FL : Fortification Level

Sample Material	FL [mg/kg]	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Grape (whole fruit)	0.01	5	100	3.1	100	2.9
	0.10	5	102	0.9	101	0.9
Orange (whole fruit)	0.01	5	90	6.3	97	6.0
	0.10	5	90	4.6	92	4.5
Lettuce (head)	0.01	5	108	6.1	108	6.2
	0.10	5	105	1.7	105	2.1
Cucumber (whole fruit)	0.01	5	97	2.1	96	1.6
	0.10	5	98	2.3	101	2.7

Avocado (whole fruit)	0.01	5	99	4.9	96	5.0
	0.10	5	87	1.9	87	1.9
Wheat (Grain)	0.01	5	87	5.5	88	5.4
	0.10	5	74	5.3	73	5.5

Table 10: Matrix effect evaluation for phosphorous acid
FL: Fortification Level

Sample Material	FL (mg/kg)	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Grape (whole fruit)	0.1	5	156	6.7	99	8.5
	1	5	131	1.9	102	2.1
Orange (whole fruit)	0.1	5	189	10.5	76	18.3
	1	5	147	2.5	93	2.3
Lettuce (head)	0.1	5	193	6.4	81	9.7
	1	5	160	3.8	104	3.9
Cucumber (whole fruit)	0.1	5	185	4.7	87	7.0
	1	5	135	2.6	100	2.2
Avocado (whole fruit)	0.1	5	139	3.7	73	5.1
	1	5	132	6.8	100	6.7
Wheat (Grain)	0.1	5	152	8.3	98	5.8
	1	5	163	2.6	79	2.5

In all the sample materials, the measurement of phosphorous acid must be established using matrix matched standards. So the measurement of both compounds is established using matrix matched standards.

3.4 Limit of Quantification and Recovery Experiments

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq 20\%$ could be obtained. The LOQ was set at 0.01 mg/kg for fosetyl-Al and at 0.1 mg/kg for phosphorous acid in grape (whole fruit), orange (whole fruit), lettuce (head), cucumber (whole fruit), avocado (whole fruit), and wheat (grain).

To validate the method for these matrices, samples were fortified with a defined amount of fosetyl-Al and phosphorous acid prior to analysis.

3.5 Recovery Rates

The detailed recovery results obtained are listed in Table 11 and Table 12.

Table 11: Recovery rates obtained for fosetyl-Al, FL: fortification level, RSD: relative standard deviation

Crop	Sample Material	FL [mg/kg]	Recovery of fosetyl-Al				
			Single Values [%]			Mean [%]	RSD [%]
Grape	Whole fruit	0.01	102	102	95	100	2.9
			101	100			
		0.1	101	102	102	101	0.9
			102	100			
			Overall Mean and RSD			101	2.1
Orange	Whole fruit	0.01	102	100	98	97	6.0
			87	97			
		0.1	96	95	94	92	4.5
			90	86			
			Overall Mean and RSD			95	5.6
Lettuce	Head	0.01	104	98	109	108	6.2
			113	114			
		0.1	102	106	104	105	2.1
			107	107			

			Overall Mean and RSD			106	4.5
Cucumber	Whole fruit	0.01	95	96	98	96	1.6
			98	95			
		0.1	106	101	101	101	2.7
			99	100			
			Overall Mean and RSD			99	3.4
Avocado	Whole fruit	0.01	104	95	92	96	5.0
			94	93			
		0.1	88	88	85	87	1.9
			89	86			
			Overall Mean and RSD			91	6.1
Wheat	Grain	0.01	86	92	93	88	5.4
			85	82			
		0.1	75	79	69	73	5.5
			71	71			
			Overall Mean and RSD			80	10.9

Table 12: Recovery rates obtained for Phosphorous acid, FL: fortification level, RSD: relative standard deviation

Crop	Sample Material	FL [mg/kg]	Recovery of Phosphorous acid				
			Single Values [%]			Mean [%]	RSD [%]
Grape	Whole fruit	0.1	96	94	113	99	8.5
			100	92			
		1	100	105	104	102	2.1
			101	101			
			Overall Mean and RSD			101	6.0
Orange	Whole fruit	0.1	65	99	70	76	18.3

			78	67			
		1	97	99	101	98	2.3
			95	99			
			Overall Mean and RSD			87	17.3
Lettuce	Head	0.1	82	94	76	81	9.7
			79	74			
		1	103	108	98	104	3.9
			106	107			
			Overall Mean and RSD			93	14.7
Cucumber	Whole fruit	0.1	83	82	82	87	7.0
			91	95			
		1	102	102	101	100	2.2
			97	99			
			Overall Mean and RSD			93	8.9
Avocado	Whole fruit	0.1	74	77	68	73	5.1
			70	75			
		1	110	97	92	100	6.7
			103	100			
			Overall Mean and RSD			87	17.8
Wheat	Grain	0.1	90	94	101	98	5.8
			102	103			
		1	77	77	81	79	2.5
			79	81			
			Overall Mean and RSD			89	12.2

The obtained recovery rates are summarised below in Table 13.

In total 60 recovery rates were determined for each compound. The single recovery rates were in the range of 69 to 114 % for fosetyl-AI and of 65 to 113 % for phosphorous acid. The mean

recovery rates per fortification level were in the range of 93 to 97 % for fosetyl-Al with an overall recovery rate over all sample materials and fortification levels of 95% and of 86 to 97 % for phosphorous acid with an overall recovery rate over all sample materials and fortification levels of 91 %.

- 5 The relative standard deviations (RSD) for the single fortification levels ranged from 7.6 to 12.3 % for fosetyl-Al and from 9.5 to 14.9 % for phosphorous acid (n=30). The overall RSD values per sample material were between 2.1 and 10.9 % for fosetyl-Al and 6.0 and 17.8 % for phosphorous acid (n=10). The RSD value across all samples was 10.2 % for fosetyl-Al and 13.7 % for phosphorous acid (n=60).

10 Table 13: Summary of the recovery data for the determination of accuracy and repeatability; RSD: relative standard deviation

	Parameter	fosetyl-Al	Phospho- rous acid
Accuracy	Single recoveries [%]	69 - 114	65 - 113
	Mean recoveries per fortification level [%]	93 - 97	86 - 97
	Mean recoveries per sample material [%]	80 - 106	87 - 101
	Overall mean [%]	95	91
	Number of values n	60	60
Repeatability	RSD per fortification level [%]	7.6 - 12.3	9.5 - 14.9
	RSD per sample material [%]	2.1 - 10.9	6.0 - 17.8
	Overall RSD [%]	10.2	13.7

3.6 Storage Stability of Extracts

The stability of final extracts containing fosetyl-Al and its metabolite phosphorous acid was determined. For this purpose, the final extracts of recovery samples were let in the auto sampler rack thermo stated at about 10 °C after initial analysis and re-analysed after a storage period of several days.

The results of the storage stabilities are detailed in Table 14.

Table 14: Stability period of final extracts in the auto sampler rack at about 10°C

Sample Material	Stability period (days)
Grape (whole fruit)	7 days
Orange (whole fruit)	4 days
Lettuce (head)	8 days
Cucumber (whole fruit)	8 days
Avocado (whole fruit)	4 days
Wheat (Grain)	3 days

4 Evaluation and Discussion

The presented residue analytical method modification 00861/M001 was validated for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in grape (whole fruit), orange (whole fruit), lettuce (head), cucumber (whole fruit), avocado (whole fruit), and wheat (grain) by LC/MS/MS.

fosetyl-Al and its metabolite (phosphorous acid) were extracted from the sample material with a mixture of acetonitrile/water (50/50). After centrifugation and dilution of the sample material, the residues are quantified by HPLC using an Hypercarb column and detected by tandem mass spectrometry with electrospray ionisation. The quantification was done by an external standardisation in matrix matched standards

The validation set included the determination of the detector linearity, the limit of quantification, the accuracy of the method and the storage stability of sample final extracts.

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at respective concentrations between 0.1 and 5 µg/L and 1 and 50 µg/L, except for wheat samples between 0.31 and 8.3 µg/L and 3.1 and 83 µg/L. The detector response was linear in these ranges.

The occurrence of matrix effects was monitored.

5 In all the sample materials, the measurement of phosphorous acid must be established using matrix matched standards. So the measurement of both compounds is established using matrix matched standards.

- 6 The apparent residues for all control samples were below 30% of the LOQ for each compound, i.e. < 0.003 mg/kg of fosetyl-Al and < 0.03 mg/kg of phosphorous acid.

7 The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq 20\%$ could be obtained. The LOQ was
10 set at 0.01 mg/kg for fosetyl-Al and at 0.1 mg/kg for phosphorous acid in grape (whole fruit), orange (whole fruit), lettuce (head), cucumber (whole fruit), avocado (whole fruit), and wheat (grain).

11 The accuracy of the method can be assessed on the basis of the determined recovery rates.
12 The single recovery rates were in the range of 69 to 114 % for fosetyl-Al and of 65 to 113 % for phosphorous acid. The mean recovery rates per fortification level were in the range of 93 to 97 % for fosetyl-Al with an overall recovery rate over all sample materials and fortification levels of 95% and of 86 to 97 % for phosphorous acid with an overall recovery rate over all sample materials and fortification levels of 91 %. The accuracy of the method fulfils the requirements for
20 residue analytical methods which demand that the mean recoveries for each fortification level should be in the range of 70-110%.

21 The precision and repeatability of the method can be assessed on the basis of the determined relative standard deviations (RSD) for the mean values of the recovery rates. The relative
25 standard deviations (RSD) for the single fortification levels ranged from 7.6 to 12.3 % for fosetyl-Al and from 9.5 to 14.9 % for phosphorous acid (n=30). The overall RSD values per sample material were between 2.1 and 10.9 % for fosetyl-Al and 6.0 and 17.8 % for phosphorous acid (n=10). The RSD value across all samples was 10.2 % for fosetyl-Al and 13.7 % for phosphorous acid (n=60).

30 The overall RSD values per sample material were between 2.1 and 10.9 % for fosetyl-Al and 6.0 and 17.8 % for phosphorous acid (n=10). The RSD value across all samples was 10.2 % for fosetyl-Al and 13.7 % for phosphorous acid (n=60). All RSD values were well below 20%, so that the precision and repeatability of the method can be considered acceptable.

All results of the method validation are in accordance with the general requirements for residue analytical methods, so that this method modification has been validated successfully.

Appendix 1
Flow Diagram of Residue Method 00861/M001

Extraction

1. Weigh 20.0 g of homogeneous sample material into a 125 mL polypropylene bottle.
2. Add 80 mL of acetonitrile / water (50/50, v/v).
3. Blend the sample using a high-speed blender (IKA or equivalent) for approx. 5 minutes.
4. Centrifuge the extract (3600 rpm – 5°C) for approx. 5 minutes.
5. Pour the supernatant into a 200 mL volumetric flask.
6. Add 80 mL of acetonitrile / water (50/50, v/v) on the bottom.
7. Blend the sample using a high-speed blender (IKA or equivalent) for approx. 5 minutes.
8. Centrifuge the extract (3600 rpm – 5°C) for approx. 5 minutes.
9. Pour the supernatant into the volumetric flask.
10. Make-up to 200 mL with methanol. This is the Extract A.
11. Centrifuge an aliquot of about 10 mL of Extract A (6000 rpm – ambient) for approx. 10 minutes.
12. Filter the extract through an Acrodisc CR 25 mm PTFE filter (0.45 µm).
13. Dilute five times the extract using acidified methanol with formic acid 0.5 %. This is the Final Extract.

Remark : for wheat samples, from stage 10, follow the preparation as described below :

10. Add 1 mL of concentrated formic acid and make-up to 200 mL with methanol. This is the Extract A.
11. Centrifuge an aliquot of about 10 mL of Extract A (6000 rpm – ambient) for approx. 10 minutes.
12. With dilutor, dilute twice the supernatant using acidified methanol with formic acid 0.5 %.
13. Filter the extract through an Acrodisc CR 25 mm PTFE filter (0.45 µm). This is the Final Extract.

HPLC Measurement with Electrospray MS/MS detection

Appendix 2
Details on LC-MS/MS conditions

5 Comment:
Synchronization Mode: LC Sync
Auto-Equilibration: Off
Acquisition Duration: 11min59sec
Number Of Scans: 888
10 Periods In File: 1
Acquisition Module: Acquisition Method
Software version: Analyst 1.4

15 MS Method Properties:

Period 1:

Scans in Period: 888
20 Relative Start Time: 0.00 msec
Experiments in Period: 1

Period 1 Experiment 1:

25 Scan Type: MRM (MRM)
Polarity: Negative
Scan Mode: N/A
Ion Source: Turbo Spray
Resolution Q1: Unit
30 Resolution Q3: Unit
Intensity Thres.: 0.00 cps
Settling Time: 0.0000 msec
MR Pause: 5.0070 msec
MCA: No
35 Step Size: 0.00 amu

Quantifier transitions:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
80.90	82.90	500.00	DP	-55.00	-55.00
40 (phosphorous acid)			CE	-38.00	-38.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
109.00	80.90	200.00	DP	-45.00	-45.00
45 (fosetyl-Al)			CE	-16.00	-16.00

Parameter Table (Period 1 Experiment 1):

CUR: 20.00
GS1: 40.00
50 GS2: 60.00
IS: -4500.00
TEM: 600.00
ihe: ON
CAD: 6.00
55 EP: -10.00
CXP: -1.00

Confirmatory transitions:

Q1 Mass (amu) Q3 Mass (amu)		Dwell(msec)	Param	Start	Stop
80.90	78.90	600.00	DP	-55.00	-55.00
(phosphorous acid)			CE	-22.00	-22.00

	Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
	109.00	63.00	200.00	DP	-45.00	-45.00
10	(fosetyl-Al)			CE	-38.00	-38.00

Parameter Table (Period 1 Experiment 1):

	CUR:	20.00
	GS1:	40.00
15	GS2:	60.00
	IS:	-4500.00
	TEM:	600.00
	ihe:	ON
	CAD:	6.00
20	EP:	-10.00
	CXP:	-5.00

Agilent 1100 LC Pump Method Properties :

25

Pump Model: Agilent 1100 LC Binary Pump

Minimum Pressure (psi): 0.0

Maximum Pressure (psi): 5801.0

Dead Volume (μl): 40.0

30

Maximum Flow Ramp (ml/min²): 100.0

Maximum Pressure Ramp (psi/sec): 290.0

Step Table.

Step	Total Time(min)	Flow Rate(μl/min)	A (%)	B (%)
0	0.00	400	65.0	35.0
35	10.00	400	65.0	35.0

Left Compressibility: 50.0

Right Compressibility: 115.0

Left Dead Volume (μl): 40.0

Right Dead Volume (μl): 40.0

40

Left Stroke Volume (μl): -1.0

Right Stroke Volume (μl): -1.0

Left Solvent: A2 (water + 0.5% formic acid)

Right Solvent: B2 (methanol)

45 CTC PAL Auto sampler Method Properties :

	Loop Volume1 (µl):	100	
	Loop Volume2 (µl):	20	
	Injection Volume (µl):	50.000	
50	Method Description:		
	Syringe:	250ul	
	Analyst LC-Inj		
	Air Volume (µl)		0
	Pre Clean with Solvent 1 ()		2
55	Pre Clean with Solvent 2 ()		1
	Pre Clean with Sample ()		0
	Filling Speed (µl/s)		50

Filling Strokes () 0
 Inject to LC Vlv2
 Injection Speed (µl/s) 50
 Pre Inject Delay (ms) 500
 5 Post Inject Delay (ms) 500
 Post Clean with Solvent 1 () 3
 Post Clean with Solvent 2 () 2
 Valve Clean with Solvent 1 () 2
 10 Valve Clean with Solvent 2 () 1

Agilent 1100 LC Pump Method Properties

Pump Model: Agilent 1100 LC Quaternary Pump
 15 Minimum Pressure (psi): 0.0
 Maximum Pressure (psi): 5801.0
 Compressibility: 100.0
 Dead Volume (µl): 40.0
 Stroke Volume (µl): -1.0
 20 Maximum Flow Ramp (ml/min²): 100.0
 Maximum Pressure Ramp (psi/sec): 290.0

Step Table:

Step	Total Time	Flow Rate	A (%)	B (%)	C (%)	D (%)	TE#1	TE#2
	TE#3 TE#4		(min)		(µl/min)		(water)	
	(methanol)							
0	0.00	200	50.0	50.0	0.0	0.0	open	open
	open							
1	10.00	200	50.0	50.0	0.0	0.0	open	open
	open							

Primary Flow Rate (µl/min): 200.0
 Flow Sensor Calibration Table Index: 0

35 Valco Valve Method Properties

Valco Valve	Divertor	Total Time (min)	Position
1	0.0 B		waste
2	3.0 A		spectro

Gas pressure		Auto sampler CTC Analytics	
		HTS Pal (n° 1303)	
N2	4 bar	Peltier rack	10 °C
Air gas	7.5 bar	Loop	20 µL
Air Exhaust gas	4 bar	Solvent 1	H ₂ O + 0.5 % HCOOH
		Solvent 2	MeOH
		Oven Agilent	
		G1316A (n°1294)	
		Temperature	Not used

		Valco valve	Column selector
		C2-0000EP V1C1 (n°1289)	C5-0006EMTD (n° 1290)
		Ten ports	6 positions
		2 positions	Not used
		A = MS B = Waste	
Pumps		Source n° 1291	Probe TurbolonSpray n° 1288
Binary pump Agilent 1100 G1312A (n° 1297)	133 bar		
Flow	0.4 mL/min	Horizontal Position	7 (x axis)
A2 = H ₂ O + 0.5% Formic Acid B2 = MeOH	65 % 35 %	Vertical Position	7 (y axis)
Isocratic		Capillary exit	1 mm
		Mass Spectrometer API 4000	
		Device GLP n° 1292	
Quaternary pump Agilent 1100 G1354A (n° 1298)			
Flow	0.2mL/min	Column	
A = H ₂ O	50 %	Precolumn	SMPREA1
B = MeOH	50 %	Column	SMAR 69-1
C =	-	Thermo Hypercarb 100 x 3.0mm 5µm	
D =	-	(ambient temperature)	
Isocratic mode			

Appendix 3
Results obtained with confirmatory transitions

Crop	Sample Material	FL [mg/kg]	Recovery of fosetyl-Al				
			Single Values [%]			Mean [%]	RSD [%]
Grape	Whole fruit	0.01	101	107	106	100	8.8
			101	85			
		0.1	98	102	93	99	4.5
			96	104			
			Overall Mean and RSD			99	6.7
Orange	Whole fruit	0.01	121	121	110	110	10.3
			106	94			
		0.1	98	102	91	94	7.0
			92	85			
			Overall Mean and RSD			102	12.2
Lettuce	Head	0.01	113	102	90	97	10.9
			93	87			
		0.1	95	97	96	96	1.4
			95	98			
			Overall Mean and RSD			97	7.4
Cucumber	Whole fruit	0.01	117	117	107	110	6.0
			103	106			
		0.1	100	100	102	100	1.4
			98	100			
			Overall Mean and RSD			105	6.6
Avocado	Whole fruit	0.01	93	94	91	92	4.9
			97	85			
		0.1	85	87	85	85	1.4
			84	84			

			Overall Mean and RSD			89	5.4
Wheat	Grain	0.01	83	88	98	89	7.3
			86				
		0.1	72	81	73	75	5.2
			72	77			
			Overall Mean and RSD			81	10.8
Grape	Whole fruit	0.1	91	86	119	96	14.1
			95	87			
		1	99	101	98	100	1.6
			100	102			
			Overall Mean and RSD			98	9.6
Orange	Whole fruit	0.1	69	92	68	74	14.1
			71	68			
		1	108	106	106	104	4.3
			97	101			
			Overall Mean and RSD			89	19.8
Lettuce	Head	0.1	91	80	94	81	14.3
			70	69			
		1	103	105	101	102	2.0
			101	100			
			Overall Mean and RSD			91	14.9
Cucumber	Whole fruit	0.1	90	93	77	86	9.9
			77	94			
		1	101	100	101	99	2.5
			95	99			
			Overall Mean and RSD			93	9.8
Avocado	Whole fruit	0.1	71	81	84	76	8.0

			70	76		
		1	113	107	103	104
			100	99		5.5
			Overall Mean and RSD			90
						17.5
Wheat	Grain	0.1	74	74	77	74
			74	71		2.9
		1	75	82	78	80
			83	80		4.0
			Overall Mean and RSD			77
						5.1

FL : Fortification Level

Example 4 :

- 6 This detailed example concerns the analysis of fosetyl-Al and of phosphorous acid using water samples. This example is a modification M001 to the Analytical Method 00931 for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in/on drinking water and surface water by LCMSMS.
- 10 The presented residue analytical method modification 00931/M001 was validated for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in drinking water and surface water by LC/MS/MS.
- 15 The sample material is treated with a cationic resin and then concentrated. The residues of fosetyl-Al and its metabolite (phosphorous acid) are quantified by HPLC using an Hypercarb column and detected by tandem mass spectrometry with electrospray ionisation. The quantification was done by an external standardisation in matrix matched standards.
- 20 The validation set included the determination of the detector linearity, the limit of quantification and the accuracy of the method.
- The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards
- The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at
- 25 concentrations between 0.2 and 20 µg/L.
- The occurrence of matrix effects was monitored. In drinking water, the measurement of fosetyl-Al must be established using matrix matched standards. The results obtained with standards in

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pure solvent for phosphorous acid in both sample materials do not always comply with European requirements. So the measurement of both compounds in drinking water and surface water is established using matrix matched standards.

The apparent residues for all control samples were below 30% of the LOQ for each compound, i.e. < 0.00003 mg/L.

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of 20% could be obtained. The LOQ was set at 0.0001 mg/L for each compounds in drinking water and surface water.

The accuracy of the method can be assessed on the basis of the determined recovery rates. The single recovery rates were in the range of 75 to 118 % for fosetyl-Al and of 72 to 117 % for phosphorous acid. The mean recovery rates per fortification level were in the range of 89 to 96 % for fosetyl-Al with an overall recovery rate over all sample materials and fortification levels of 93% and of 91 to 93 % for phosphorous acid with an overall recovery rate over all sample materials and fortification levels of 92 %. The accuracy of the method fulfils the requirements for residue analytical methods which demand that the mean recoveries for each fortification level should be in the range of 70-110%.

The precision and repeatability of the method can be assessed on the basis of the determined relative standard deviations (RSD) for the mean values of the recovery rates. The relative standard deviations (RSD) for the single fortification levels ranged from 9.6 to 14.3 % for fosetyl-Al and from 8.8 to 19.8 % for phosphorous acid (n=10).

The overall RSD values per sample material were between 7.5 and 9.7 % for fosetyl-Al and 9.3 and 12.4 % for phosphorous acid (n=10). The RSD value across all samples was 12.7 % for fosetyl-Al and 15.0 % for phosphorous acid (n=20). All RSD values were well below 20%, so that the precision and repeatability of the method can be considered acceptable.

All results of the method validation are in accordance with the general requirements for residue analytical methods, so this method modification has been validated successfully.

A variant method was also validated on drinking water and surface water. The sample preparation of the main method was strongly simplified by the suppression of the concentration step. The variant method was successfully validated, according to the European requirements (96/46/EC of 16th July 1996), for non concentrated drinking water and surface water at 0.1 µg/L for fosetyl-Al and ten times this limit for fosetyl-Al and at 1 µg/L for phosphorous acid when matrix matched standards were used, independently of the LC conditions tested.

Regarding to the main method, the sample preparation was greatly simplified and allows to save a lot of time.

1. Introduction

Fosetyl-Al is a fungicide

The method modification 00931/M001 presented in this report was validated in order to suppress the steps of derivatization and clean up, to change the analysis and detection modes and to decrease the Limit of Quantification (LOQ) of original method 00931 from 0.001 mg/L for fosetyl-Al and 0.004 mg/L for phosphorous acid in surface water and from 0.002 mg/L for phosphorous acid in drinking water to 0.0001 mg/L for each compound in both sample materials.

Table 15: LOQ and principle of analytical determination

Compound		fosetyl-Al	Phosphorous acid
Determined as		fosetyl-Al	Phosphorous acid
Calculated as		fosetyl-Al	Phosphorous acid
Principle of Determination		LC/MS/MS	LC/MS/MS
LOQ [mg/L]	Drinking water	0.0001	0.0001
	Surface water	0.0001	0.0001

1.1. Citation of the Original Method

Original Method : 00931

Compounds : Fosetyl-Al and its metabolite (phosphorous acid)

Author : L. Baudet – R. Diot – M. Guillet – J.L. Kieken
Aventis CropScience Centre de Recherche de la Dargoire
14/20 rue Pierre Baizet 69009 Lyon - France

Citation : Agradoc Numbers : R011760 and R011761 dated May 22, 2000

Reason for Modification :

- Suppress the steps of derivatization and clean up.
- Change analysis and detection modes from GC/FPD to LC/MS/MS.
- Decrease the LOQ from 0.001 mg/L for fosetyl-Al and 0.004 mg/L for phosphorous acid in surface water and from 0.002 mg/L for phosphorous acid in drinking water to 0.0001 mg/L for each compound in both sample materials.

1.2. Physical and Chemical Properties

See example 3

2. Experimental Section

2.1. Materials

2.1.1. Apparatus

Standard laboratory glass consumable should be cleaned with only detergents containing no phosphate and rinsed with water and acetone.

5 To avoid any contamination, the use of disposable laboratory consumable is strongly advised.

• Balances :

➤ accuracy ± 0.1 mg (analytical standards) (e.g. Mettler AT261 range)

➤ accuracy ± 0.1 g (samples) (e.g. Mettler PM6000)

- Dilutor (e.g. Hamilton MicroLab 1000)
- 10 • Rotary shaker (e.g. Heidolph REAX 2)
- Sample Concentrator (e.g. Techne dri-block)
- Ultrasonic bath
- HPLC (e.g. Binary Pump Agilent 1100)
- Auto sampler (e.g. CTC Analytics HTC PAL)
- 15 • Triple Quadrupole HPLC-MS/MS Mass Spectrometer (e.g. Sciex Instruments, API 4000 System)
- Column (e.g. Hypercarb, 100 x 2.0 mm, 5 μ m)

2.1.2. Reagents and Supplies

- Acetone (e.g. SupraSolv® Merck)
- Methanol (e.g. SupraSolv® Merck)
- 20 • Formic acid (e.g. Normapur Prolabo)
- Cationic resin (AG 50W-X8, 20-50 Meshs, hydrogen form) (e.g. Bio Rad)
- GF/A filters (125mm), (e.g. Whatman)
- Polypro bottles (125 mL, wide opening) (e.g. Nalgen)
- Polypro bottles (1000 mL, wide opening) (e.g. Nalgen)
- 25 • Pyrex glass test tubes (20 x 150mm) (e.g. Corning)
- Polypro funnels (67mm) (e.g. Marylands Plastics)
- Glass disposable pipettes (5 mL)
- Solvent for dilution Water with 0.5 % HCOOH
- Mobile phase solvent: Water with 2 % HCOOH
- 30 Methanol with 2 % HCOOH

2.1.3. Reference Item

See example 3.

2.1.4. Standard Solutions

Stock and standard solutions were stored protected from light in a refrigerator at around 5°C.

35 Stock solutions (1000 mg/L)

65

Into a 100 mL amber screw-cap flask, weigh accurately between 20 and 50 mg of reference item. Using a burette, add a volume of water to obtain a stock solution of exactly 1000 mg/L. Mix thoroughly until complete dissolution using a magnetic stirrer. Two separate stock solutions must be prepared for each compound. After comparison of these two stock solutions, they are
5 mixed.

Mixture solution

Pipette 5 mL of each stock solution using a class "A" pipette. Pour into a class "A" 50 mL volumetric flask. Adjust volume with water, cap and mix by shaking (100 mg/L of each compound)

10 Fortifying standard solutions

By serial dilutions of the mixture solution in water, prepare the fortifying standard solution used for recoveries at 10LOQ level (0.1 mg/L of each compound) and the fortifying standard solution used for recoveries at LOQ level (0.01 mg/L of each compound).

Intermediate standard solution

15 By serial dilutions of the mixture solution, prepare the intermediate standard solution at 1.0 mg/L using water.

Standard solutions in solvent

To obtain the standard solutions used for calibration, dilute extemporaneously using a dilutor and water with 0.5% formic acid, the intermediate standard solution to obtain the following
20 concentrations: 0.2, 0.4, 0.5, 0.75, 1, 2, 5, 10 and 20 µg/L.

Matrix matched standard solutions used for calibration

The occurrence of matrix effects was monitored and the measurement of both compounds is established using matrix matched standards in both sample materials.

From the intermediate standard solution, the dilutions are the same as preparation of standard
25 solutions in solvent, except the dilution mixture which is the final extract of a control sample.

Remarks :

- 20 to 25 mL of final extract are necessary to make all dilutions.
- final extracts of control sample could be prepared a day before use and stocked in a refrigerator.

30

2.1.5. Stability of the standard solutions

The stock solutions, stored protected from light in a refrigerator at around 5°C, were found to be stable for 9 months and a half.

2.2. Residue Analytical Methodology

Some modifications compared to the original analytical method were introduced.

The method modification 00931/M001 presented in this report was validated in order to suppress the steps of derivatization and clean up, to change the analysis and detection modes and to decrease the Limit of Quantification (LOQ) of original method 00931 from 0.001 mg/L for fosetyl-Al and 0.004 mg/L for phosphorous acid in surface water and from 0.002 mg/L for phosphorous acid in drinking water to 0.0001 mg/L for each compound in both sample materials.

- The Limit of Quantification was decreased from 0.001 mg/L for fosetyl-Al and 0.004 mg/L for phosphorous acid in surface water and from 0.002 mg/L for phosphorous acid in drinking water to 0.0001 mg/L for each compound in both sample materials.
- The NaOH treatment was replaced by a resin treatment.
- The derivatization step was suppressed.
- The clean-up step with liquid-liquid partition was suppressed.
- The quantification was carried out by LC/MS/MS instead of GC/FPD.

All modifications were included in the description below.

A flow chart of the method is given in Appendix 1.

Note : during analysis, for each sample set, it is necessary to do a blank reagent where water sample is replaced by Milli Q water to be sure that no phosphorous acid contamination (< 30% LOQ) coming from sample preparation is found.

Remark : standard laboratory glass consumable should be cleaned with only detergents containing no phosphate and rinsed with water and acetone.

To avoid any contamination, the use of disposable laboratory consumable is strongly advised.

For recovery experiments, samples are fortified by adding the appropriate standard solution to the sample material after weighing and before shaking.

Preparation of the cationic resin :

15. Weigh about 25 g of AG 50W-X8 resin into a 1000 mL polypropylene bottle.

16. Add about 500 mL of water.

17. Shake using a rotary shaker for about 10 minutes.

18. Discard the supernatant water.

19. Do steps 2 to 4 a second time.

20. Do steps 2 to 4 a third time.

21. Filtrate residual water and resin through two GF/A filters put in a polypropylene funnel and previously rinsed with water.

Remark: resin is prepared either just before use or in advance, stored at ambient temperature and re-hydrated just before use.

Caution: phosphorous acid interferences could be observed if the resin is not washed as described above.

Samples preparation :

1. Weigh 0.6 g of AG 50W-X8 resin previously washed into a 125 mL polypropylene bottle.
2. Using disposable Pasteur pipette, weigh 20.0 g of homogeneous sample material into the bottle
Note: weight of the sample is used for residue calculation, addressed as variable W_{Water}
3. Shake the sample using a rotary shaker for 10 minutes. This is the Extract A.
Note: The weight of Extract A is used for residue calculation, addressed as variable W_{Extract}
In this case, there was no extraction: no solvent was added to water sample; it was just a resin treatment, so $W_{\text{Extract}} = W_{\text{Water}} = 20 \text{ g}$
4. Using a disposable glass pipette, transfer an aliquot of 5 mL of supernatant into a test tube previously weighted.
Note: The volume of aliquot is used for residue calculation, addressed as variable V_{Aliquot} (Instead of transferring 5 mL, this aliquot could also be done by weighing 5 g = W_{Aliquot})
5. Evaporate under a nitrogen flow using a sample concentrator at a temperature of about 60°C to approximately 0.5 g. Caution not to evaporate to dryness.
6. Using disposable pipette make-up to 1.0 g with acidified water with formic acid 0.5 %.
Note: the concentration of formic acid in acidified water used in step 6 is a critical parameter: it has an important action on the H_3PO_3 peak shape obtained in LC. The increase from 0.5% to 2% of formic acid in acidified water can give a very large H_3PO_3 peak, and accordingly a loss of sensitivity.
7. Sonicate for about 5 minutes. This is the Final Extract.
Note: weight of Final Extract is used for residue calculation, addressed as variable W_{End}
8. Proceed to LC/MS/MS measurement, Chapter 2.3.
Note: if it is necessary to dilute the Final Extract due to a concentration outside the calibration curve: use final extract of control sample because matrix matched standards are used for calibration.

2.3. Analysis and Instrument Conditions

The final extracts are injected into a high performance liquid chromatograph and detected by tandem mass spectrometry with electrospray ionisation.

The quantification is carried out by external standardisation using matrix matched standards.

Exemplary LC/MS/MS conditions that were used in the course of this method validation are listed in chapters 2.3.1 and 2.3.2. These conditions are given as a guidance and may have to be adapted for other HPLC-MS/MS systems

2.3.1. HPLC Conditions

Instrument: Binary pump Agilent 1100

Auto sampler: CTC Analytics HTS PAL

Column: Hypercarb, 100 x 2.0 mm, 5 µm

Precolumn: none

Injection Volume: 50 µL

Column temperature: ambient (about 25°C)

5 Mobile Phase:

Isocratic mode: 55 / 45 (v/v) Methanol + 2% formic acid / water + 2% formic acid

Flow (Column): 200 µL/min

Retention Times: from 3.1 to 4.1 min for phosphorous acid and from 3.9 to 5.3 min for fosetyl-Al.

10

Remarks :

- Retention times in concentrated samples are lightly shorter than in solvent (about 0.2 min in surface water and 0.5 min in drinking water).
- It is necessary to wait about 2 hours the stabilisation of the HPLC system before injecting. During a samples set, a light drift of retention time of both compounds can be observed.
- Hypercarb precolumn must not be used.
- Depending of the phase batch of Hypercarb column, the percentage of formic acid in the mobile phase could be adapted to improve peaks shape (from 0.5% to 2%).

15

20

2.3.2 MS/MS Conditions

See example 3.

2.3.3. Confirmatory transitions

See example 3.

Note : all recovery samples were also analysed using confirmatory transitions.

25

The results are given in Appendix 6.

2.4. Linearity of the Detector

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

30

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at concentrations between 0.2 and 20 µg/L.

Table 13: Standard concentrations prepared for the determination of detector linearity. The concentration corresponding to the LOQ is printed in the third column of the table.

HPLC-MS/MS	Standard Concentrations [µg/L]								
fosetyl-Al	0.2	0.4	0.5	0.75	1	2	5	10	20
phosphorous acid	0.2	0.4	0.5	0.75	1	2	5	10	20

2.5. Storage Stability of Extracts

- 5 The stability of sample extracts containing fosetyl-Al and phosphorous acid was not determined in this study.

2.6. Calculation

2.6.1. Calculation of Residues

10

Evaluation in this case is performed according to the external standardisation using matrix matched standards.

During the analysis of each set of samples, the 9 standard solutions mentioned in Table 16 are injected once. Standards should be interspersed with samples to compensate for
15 any minor change in instrument response.

For each compound, the peak area is plotted versus the concentration in order to establish a calibration curve obtained by linear regression weighting 1/x with least squares method.

The corresponding model to determine the concentration in final extracts is calculated using the Analyst Software (Version 1.4).

- 20 Each final extract is injected once using the same conditions as previously described for the standard solutions.

Using the predicting mathematical model previously established, the final concentration in µg/L of each compound is determined for each injection.

- 25 For each compound, the amount of residue R, expressed in mg/L is calculated, using the following formula :

$$R = \frac{C \times V_{\text{End}} \times V_{\text{Extract}}}{1000 \times V_{\text{Water}} \times V_{\text{Aliquot}}}$$

- where: R : Determined amount of residue of fosetyl-Al or phosphorous acid in **mg/L**
- C : Concentration of fosetyl-Al or phosphorous acid found in the final extract in **µg/L**
- V_{End} : Volume of the final extract in **mL**, considering that : $V_{\text{End}} = W_{\text{End}} \times \text{Density}^{-1}$
 where W_{End} = weight of the final extract in g, here 1 g and $\text{Density}^{-1} = 1 \text{ mL/g}$ (*)
- V_{Extract} : Volume of the extract A in **mL**, considering that : $V_{\text{Extract}} = W_{\text{Extract}} \times \text{Density}^{-1}$
 where W_{Extract} = weight of the extract A in g, here 20 g and $\text{Density}^{-1} = 1 \text{ mL/g}$ (*).
 In this case, there was no extraction: no solvent was added to water sample; it was just a resin treatment, so $W_{\text{Extract}} = W_{\text{Water}} = 20 \text{ g}$
- V_{Water} : Volume of the analytical sample in **mL**, considering that : $V_{\text{Water}} = W_{\text{Water}} \times \text{Density}^{-1}$
 where W_{Water} = weight of the water in g, here 20 g and $\text{Density}^{-1} = 1 \text{ mL/g}$ (*)
- V_{Aliquot} : Aliquot (of extract A) used before evaporation in **mL**, here 5 mL
 If the aliquot has been weighed, $V_{\text{Aliquot}} = W_{\text{Aliquot}} \times \text{Density}^{-1}$
 where W_{Aliquot} = weight of the aliquot in g, here 5 g and $\text{Density}^{-1} = 1 \text{ mL/g}$ (*)

5 Remarks (*):

- The density of all liquid samples (water, extracts) is considered equal to 1 independently to room temperature. This allows to convert weight of liquid samples into volume.
- In order to express phosphorous acid in fosetyl-Al equivalent, the ratio of the molecular weight must be used :

10
$$\text{Ratio} = \frac{354.1 \text{ g/mol}}{(82.0 \times 3) \text{ g/mol}} = 1.44$$

because 1 mole fosetyl-Al (354.1g) gives 3 moles of phosphorous acid.

2.6.2. Calculation of Recovery Rates

The concentration of each compound in µg/L is determined for the recovery sample according to 2.6.1.

The percent recovery rate is then calculated as follows :

$$\text{Rec} = \frac{C \times 100}{A}$$

- where: Rec : Recovered amount found in fortified sample in %
- C : Concentration of fosetyl-Al or phosphorous acid found in the analysed extract in µg/L
- A : Fortified amount of fosetyl-Al or phosphorous acid in µg/L

2.6.3. Calculation of Relative Standard Deviation (RSD)

The RSD is calculated as follows :

RSD (%) = S.D. / Mean Recovery x 100 %

$$S.D. = \left[\frac{\sum (R_i - R_m)^2}{n - 1} \right]^{1/2}$$

- R_i : recovery
- R_m : mean recovery
- n : number of recoveries

3 Results and Discussion

3.1. Specificity and Selectivity

The method allows the determination of fosetyl-Al and its metabolite (phosphorous acid) in drinking water and surface water samples.
The specificity of the method resulted from the HPLC separation in combination with the very selective MS/MS detection.

3.2. Apparent residues in Control Samples

Two control samples were analysed for each sample material. The origin of the control materials used is listed in Table 17.

Table 17: Origin of untreated control samples

Test system	Origin
Drinking water	BCS - CRLD - Tap water from lab D221 - Lyon - France
Surface water	St Agnan en Vercors - France

Some characteristics of surface water are recorded in Table 18.

Table 18: Characteristics of surface water

date of collect (yyyy/mm/dd)	pH at 16.5 °C (NF T 90-008)	Ca ⁺⁺ (mg/L) (potentiometric detection)	clay particles (mg/L) (NF EN 872)	total organic carbon (TOC) (mg/L) (NF EN 1484)	conductivity (µS/cm) (NF EN 27 888)
2005/03/27	8.1	88	< 2	1.00	400

These characteristics were determined by :
Cemagref
Division qualité des eaux et prévention des pollutions
Groupement de Lyon
3 bis quai Chauveau
69336 Lyon cedex 09 - France

A residue level estimation in control samples was performed. The results are listed in Table 19.
The apparent residues for all control samples were below 30% of the LOQ for each compound,
i.e. < 0.00003 mg/L.

Table 19: Apparent residues in untreated control samples for fosetyl-Al and phosphorous acid

Sample Material	Origin	LOQ [mg/L]		Apparent residues [mg/L]	
		fosetyl-Al	phosphorous acid	fosetyl-Al	phosphorous acid
Drinking water	BCS - CRLD - Tap water from lab D221 - Lyon - France	0.0001	0.0001	< 30% LOQ	< 30% LOQ
Surface water	St Aignan en Vercors - France	0.0001	0.0001	< 30% LOQ	< 30% LOQ

3.3. Limit of detection

Control sample fortified at 0.00005 mg/L were analysed to test the limit of detection of fosetyl-Al and phosphorous acid. The results are given in Table 20.

Table 20: Recovery rate obtained for fosetyl-Al and phosphorous acid at 0.05 µg/L fortification level

Sample Material	Fortification level [mg/L]		Recovery [%]	
	fosetyl-Al	phosphorous acid	fosetyl-Al	phosphorous acid
Drinking water	0.00005	0.00005	89	75
Surface water	0.00005	0.00005	106	95

3.4. Linearity of the Detectors and Matrix Effects

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

- 15 The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at concentrations between 0.2 and 20 µg/L.

Experimental details can be found in Chapter 2.4.

- 10 In each chromatogram, the measured peak area of fosetyl-Al or phosphorous acid is plotted versus the corresponding concentration of respectively fosetyl-Al or phosphorous acid contained in each standard solution, in order to obtain calibration curve of the form :

$$y = ax + b \quad (1/x \text{ weighting})$$

where : y = peak area,

x = concentration in injected standard solution

- 15 The results of the determination of detector response for LC/MS/MS are summarised in Table 21.

Table 21: Summary of the determination of detector linearity for LC/MS/MS.

Detection	Parameter	fosetyl-Al	phosphorous acid
LC/MS/MS	Linear range [µg/L]	0.2 - 20	0.2 - 20
	No. of concentrations	9	9
	No. of injections	1	1
	Model	1/x weighted linear regression	1/x weighted linear regression
	Correlation coefficient (R) for standards prepared in solvent	> 0.9993	> 0.9991
	Correlation coefficient (R) for matrix matched standards	> 0.9981	> 0.9990

An excellent linear correlation between the injected amount of the analytes and the detector responses of LC/MS/MS was observed for standards in the range of 0.2 to 20 µg/L for both compounds, using either standards prepared in solvent or matrix matched standards.

- 5 The occurrence of matrix effects was monitored. The results are shown in Table 22 and table 23.

Table 22: Matrix effect evaluation for fosetyl-Al.

FL : Fortification Level

Sample Material	FL [mg/L]	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Drinking water	0.0001	5	25	17.0	86	12.2
	0.001	5	33	7.0	82	6.5
Surface water	0.0001	5	97	7.9	107	6.4
	0.001	5	103	3.4	96	3.3

Table 23: Matrix effect evaluation for phosphorous acid

FL : Fortification Level

Sample Material	FL [mg/L]	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Drinking water	0.0001	5	80	3.3	76	5.7
	0.001	5	64	11.3	88	11.9
Surface water	0.0001	5	123	4.2	109	4.1
	0.001	5	101	4.6	93	4.6

In drinking water, the measurement of fosetyl-Al must be established using matrix matched standards. The results obtained with standards in pure solvent for phosphorous acid in both

An excellent linear correlation between the injected amount of the analytes and the detector responses of LC/MS/MS was observed for standards in the range of 0.2 to 20 µg/L for both compounds, using either standards prepared in solvent or matrix matched standards.

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Table 22: Matrix effect evaluation for fosetyl-Al.

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			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Drinking water	0.0001	5	25	17.0	86	12.2
	0.001	5	33	7.0	82	6.5
Surface water	0.0001	5	97	7.9	107	6.4
	0.001	5	103	3.4	96	3.3

Table 23: Matrix effect evaluation for phosphorous acid
FL : Fortification Level

10

Sample Material	FL [mg/L]	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Drinking water	0.0001	5	80	3.3	76	5.7
	0.001	5	64	11.3	88	11.9
Surface water	0.0001	5	123	4.2	109	4.1
	0.001	5	101	4.6	93	4.6

In drinking water, the measurement of fosetyl-Al must be established using matrix matched standards. The results obtained with standards in pure solvent for phosphorous acid in both

sample materials do not always comply with European requirements. So the measurement of both compounds in drinking water and surface water is established using matrix matched standards.

3.5. Limit of Quantification and Recovery Experiments

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq 20\%$ could be obtained. The LOQ was set at 0.0001 mg/L for each compounds in drinking water and surface water.

To validate the method for these matrices, samples were fortified with a defined amount of fosetyl-Al and phosphorous acid prior to analysis.

3.6. Recovery Rates

The detailed recovery results obtained are listed in Table 24 and table 25.

Table 24: Recovery rates obtained for fosetyl-Al, RSD: relative standard deviation

Sample Material	Fortification Level [mg/L]	Recovery of fosetyl-Al				
		Single Values [%]			Mean [%]	RSD [%]
Drinking water	0.0001	76	76	87	86	12.2
		90	101			
	0.001	78	75	87	82	6.5
		87	82			
		Overall Mean and RSD			84	9.7
Surface water	0.0001	100	103	107	107	6.4
		118	106			
	0.001	98	100	96	96	3.3
		92	94			
		Overall Mean and RSD			101	7.5

Table 25: Recovery rates obtained for Phosphorous acid, RSD: relative standard deviation

Sample Material	Fortification Level [mg/L]	Recovery of Phosphorous acid				
		Single Values [%]			Mean [%]	RSD [%]
Drinking water	0.0001	72	72	74	76	5.7
		78	82			
	0.001	80	76	89	88	11.9
		96	101			
			Overall Mean and RSD			82
Surface water	0.0001	109	106	106	109	4.1
		117	109			
	0.001	97	98	94	93	4.6
		88	90			
			Overall Mean and RSD			101

5 The obtained recovery rates are summarised below in Table 26.

In total 20 recovery rates were determined for each compound.

The single recovery rates were in the range of 75 to 118 % for fosetyl-Al and of 72 to 117 % for phosphorous acid. The mean recovery rates per fortification level were in the range of 89 to 96 % for fosetyl-Al with an overall recovery rate over all sample materials and fortification levels of 93% and of 91 to 93 % for phosphorous acid with an overall recovery rate over all sample materials and fortification levels of 92 %.

The relative standard deviations (RSD) for the single fortification levels ranged from 9.6 to 14.3 % for fosetyl-Al and from 8.8 to 19.8 % for phosphorous acid (n=10). The overall RSD values per sample material were between 7.5 and 9.7 % for fosetyl-Al and 9.3 and 12.4 % for phosphorous acid (n=10). The RSD value across all samples was 12.7 % for fosetyl-Al and 15.0 % for phosphorous acid (n=20).

Table 26: Summary of the recovery data for the determination of accuracy and repeatability;
RSD: relative standard deviation

	Parameter	fosetyl-Al	Phosphorous acid
Accuracy	Single recoveries [%]	75 - 118	72 - 117
	Mean recoveries per fortification level [%]	89 - 96	91 - 93
	Mean recoveries per sample material [%]	84 - 101	82 - 101
	Overall mean [%]	93	92
	Number of values n	20	20
Repeatability	RSD per fortification level [%]	9.6 - 14.3	8.8 - 19.8
	RSD per sample material [%]	7.5 - 9.7	9.3 - 12.4
	Overall RSD [%]	12.7	15.0

3.7. Storage Stability of Extracts

- 5 The stability of sample extracts containing fosetyl-Al and phosphorous acid was not determined in this study.

4. Evaluation and Discussion

10 The presented residue analytical method modification 00931/M001 was validated for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in drinking water and surface water by LC/MS/MS.

15 The sample material is treated with a cationic resin and then concentrated. The residues of fosetyl-Al and its metabolite (phosphorous acid) are quantified by HPLC using an Hypercarb column and detected by tandem mass spectrometry with electrospray ionisation. The quantification was done by an external standardisation in matrix matched standards.

The validation set included the determination of the detector linearity, the limit of quantification and the accuracy of the method.

- 20 The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at respective concentrations between 0.1 and 5 µg/L and 1 and 50 µg/L, except for wheat samples between 0.31 and 8.3 µg/L and 3.1 and 83 µg/L. The detector response was linear in these ranges.

- 5 The occurrence of matrix effects was monitored.

In all the sample materials, the measurement of phosphorous acid must be established using matrix matched standards. So the measurement of both compounds is established using matrix matched standards.

- 10 The apparent residues for all control samples were below 30% of the LOQ for each compound, i.e. < 0.003 mg/kg of fosetyl-Al and < 0.03 mg/kg of phosphorous acid.

- 15 The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq 20\%$ could be obtained. The LOQ was set at 0.01 mg/kg for fosetyl-Al and at 0.1 mg/kg for phosphorous acid in grape (whole fruit), orange (whole fruit), lettuce (head), cucumber (whole fruit), avocado (whole fruit), and wheat (grain).

- The accuracy of the method can be assessed on the basis of the determined recovery rates.
- 20 The single recovery rates were in the range of 69 to 114 % for fosetyl-Al and of 65 to 113 % for phosphorous acid. The mean recovery rates per fortification level were in the range of 93 to 97 % for fosetyl-Al with an overall recovery rate over all sample materials and fortification levels of 95% and of 86 to 97 % for phosphorous acid with an overall recovery rate over all sample materials and fortification levels of 91 %. The accuracy of the method fulfils the requirements for
- 25 residue analytical methods which demand that the mean recoveries for each fortification level should be in the range of 70-110%.

The precision and repeatability of the method can be assessed on the basis of the determined relative standard deviations (RSD) for the mean values of the recovery rates.

- the relative standard deviations (RSD) for the single fortification levels ranged from 7.6 to 12.3
- 30 % for fosetyl-Al and from 9.5 to 14.9 % for phosphorous acid (n=30).

The relative standard deviations (RSD) for the single fortification levels ranged from 7.6 to 12.3 % for fosetyl-Al and from 9.5 to 14.9 % for phosphorous acid (n=30). The overall RSD values per sample material were between 2.1 and 10.9 % for fosetyl-Al and 6.0 and 17.8 % for phosphorous acid (n=10). The RSD value across all samples was 10.2 % for

fosetyl-Al and 13.7 % for phosphorous acid (n=60). All RSD values were well below 20%, so that the precision and repeatability of the method can be considered acceptable.

All results of the method validation are in accordance with the general requirements for residue analytical methods, so this method modification has been validated successfully.

Note : during analysis, for each sample set, it is necessary to do a blank reagent where water sample is replaced by Milli Q water to be sure that no phosphorous acid contamination (< 30% LOQ) coming from sample preparation is found.

Remarks :

- standard laboratory glass consumable should be cleaned with only detergents containing no phosphate and rinsed with water and acetone. To avoid any contamination, the use of disposable laboratory consumable is strongly advised.
- Before start of validation / measurements equipment / chemicals should be tested for any residues of phosphorous acid.
- If some contamination is observed, see if the use of special HPLC vials (e.g. polypropylene vials, Agilent, art. 5182-0567) can be helpful to reduce the background for phosphorous acid.

Assessment of different LC conditions :

All recovery samples were also analysed using different LC conditions. The only modification was the mobile phase.

LC conditions	Original	Substitute
Change in the mobile phase composition	Isocratic mode: 55 / 45 (v/v) methanol + 2% HCOOH / water + 2% HCOOH	Isocratic mode: 35 / 65 (v/v) methanol / water + 2% HCOOH
Retention times	from 3.1 to 4.1 min for phosphorous acid and 3.9 to 5.3 min for fosetyl-Al	about 3.2 min for phosphorous acid and 5.2 min for fosetyl-Al

Complete original LC conditions are indicated in 2.3.1 .

The results are presented in Table 27.

Table 27: Summary of the results obtained with substitute LC conditions

Test system	Compound	LC conditions	Calibration curve	
			Standards in pure solvent	Matrix matched standards
Drinking water	fosetyl-Al	original	(LOQ and 10LOQ) : No	(LOQ and 10LOQ) : OK
		substitute	(LOQ and 10LOQ) : No	(LOQ and 10LOQ) : OK

	H_3PO_3	original	LOQ : OK 10LOQ : No	(LOQ and 10LOQ) : OK
		substitute	(LOQ and 10LOQ) : No	(LOQ and 10LOQ) : OK
Surface water	fosetyl-Al	original	(LOQ and 10LOQ) : OK	(LOQ and 10LOQ) : OK
		substitute	(LOQ and 10LOQ) : No	(LOQ and 10LOQ) : No
	H_3PO_3	original	LOQ : No 10LOQ : OK	(LOQ and 10LOQ) : OK
		substitute	LOQ : No 10LOQ : OK	(LOQ and 10LOQ) : OK

"OK" means that the results comply with European requirements.

"No" means that the results do not comply with European requirements.

The column "Standards in pure solvent" is given as information.

- 3 When matrix matched standards are used, the different experiments done showed that for surface water the validation failed only for fosetyl-Al at LOQ and 10LOQ if substitute LC conditions are used. For drinking water, no problem was observed.

- 10 For drinking water, the method was successfully validated, according to the European requirements (96/46/EC of 16th July 1996) at 0.1 µg/L and ten times this limit for fosetyl-Al and phosphorous acid, only when matrix matched standards were used, independently of LC conditions tested.

- 15 For surface water, the method was successfully validated, according to the European requirements (96/46/EC of 16th July 1996) at 0.1 µg/L and ten times this limit for fosetyl-Al and phosphorous acid, only when matrix matched standards were used, depending on LC conditions tested (notably for fosetyl-Al).

5. Variant method

- 20 A variant method was also validated on drinking water and surface water. The sample preparation of the main method was strongly simplified by the suppression of the concentration step and all recovery samples were analysed in the HPLC and detection conditions given in 2.3.1 and 2.3.2. before the concentration step.

A flow chart of the variant method is given in Appendix 8.

A summary of the results is given in Table 28 and the detailed recovery results obtained are listed in Appendix 7.

5 Table 28: Summary of the results obtained with variant method

Test system	Compound	LC conditions	Calibration curve	
			Standards in pure solvent	Matrix matched standards
Drinking water	fosetyl-Al	original	(LOQ and 10LOQ) : OK	(LOQ and 10LOQ) : OK
		substitute	(LOQ and 10LOQ) : No	(LOQ and 10LOQ) : OK
	H ₃ PO ₃	original	LOQ : No 10LOQ : OK	LOQ : No 10LOQ : OK
		substitute	(LOQ and 10LOQ) : No	LOQ : No 10LOQ : OK
Surface water	fosetyl-Al	original	(LOQ and 10LOQ) : OK	(LOQ and 10LOQ) : OK
		substitute	(LOQ and 10LOQ) : OK	(LOQ and 10LOQ) : OK
	H ₃ PO ₃	original	LOQ : No 10LOQ : OK	LOQ : No 10LOQ : OK
		substitute	LOQ : No 10LOQ : OK	LOQ : No 10LOQ : OK

"OK" means that the results comply with European requirements.

"No" means that the results do not comply with European requirements.

The column "Standards in pure solvent" is given as information.

- 10 Remark : to quantify non concentrated water samples, it is necessary to adapt standards solutions concentrations. As phosphorous acid is not sensitive enough, this method does not allow its determination at the limit of quantification of 0.1µg/L.

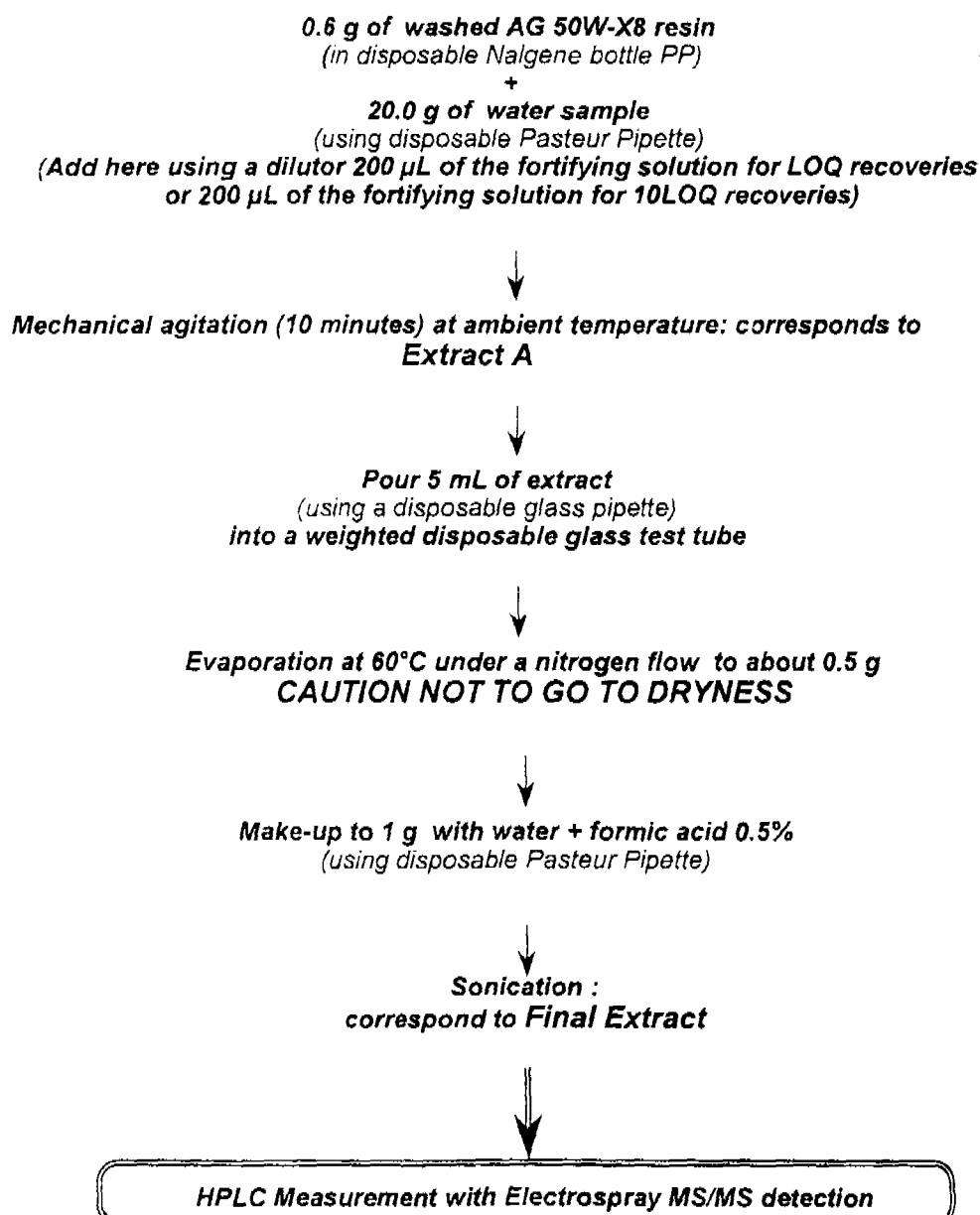
- 15 The variant method was successfully validated, according to the European requirements (96/46/EC of 16th July 1996), for non concentrated drinking water and surface water at 0.1µg/L for fosetyl-Al and ten times this limit for fosetyl-Al and at 1µg/L for phosphorous acid when matrix matched standards were used, independently of the LC conditions tested.

- 20 Regarding to the main method, the sample preparation was greatly simplified and allows to save a lot of time.

Appendix 4

Flow Diagram of Residue Method 00931/M001

Main point : for each set of samples, it is necessary to do a blank reagent where water sample is replaced by H₂O milliQ to be sure that no H₃PO₃ contamination (< 30% LOQ) coming from sample preparation is found.



If necessary to dilute the Final Extract due to concentration outside the calibration curve : use final extract of control sample

Appendix 5
Details on LC-MS/MS conditions

5 Comment:
Synchronization Mode: LC Sync
Auto-Equilibration: Off
Acquisition Duration: 10min1sec
Number Of Scans: 455
10 Periods In File: 1
Acquisition Module: Acquisition Method
Software version Analyst 1.4

MS Method Properties:

15

Period 1:

Scans in Period: 455
Relative Start Time: 0.00 msec
20 Experiments in Period: 1

Period 1 Experiment 1:

Scan Type: MRM (MRM)
25 Polarity: Negative
Scan Mode: N/A
Ion Source: Turbo Spray
Resolution Q1: Unit
Resolution Q3: Unit
30 Intensity Thres.: 0.00 cps
Settling Time: 0.0000 msec
MR Pause: 5.0070 msec
MCA: No
Step Size: 0.00 amu

35

Quantifier transitions:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
80.90	78.90	400.00	DP	-55.00	-55.00
(phosphorous acid)			CE	-22.00	-22.00
			CXP	-5.00	-5.00

40

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
108.90	80.90	200.00	DP	-45.00	-45.00
(fosetyl-Al)			CE	-16.00	-16.00
			CXP	-1.00	-1.00

45

Confirmatory transitions:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
80.90	62.90	400.00	DP	-55.00	-55.00
(phosphorous acid)			CE	-38.00	-38.00
			CXP	-1.00	-1.00

50

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
108.90	62.90	300.00	DP	-45.00	-45.00
(fosetyl-Al)			CE	-38.00	-38.00
			CXP	-5.00	-5.00

55

Parameter Table (Period 1 Experiment 1):

CAD: 6.00
 CUR: 20.00
 5 GS1: 40.00
 GS2: 60.00
 TEM: 600.00
 ihe: ON
 IS: -4500.00
 10 EP: -10.00

Agilent 1100 LC Pump Method Properties :

Pump Model: Agilent 1100 LC Binary Pump
 15 Minimum Pressure (psi): 0.0
 Maximum Pressure (psi): 5801.0
 Dead Volume (μl): 40.0
 Maximum Flow Ramp (ml/min²): 100.0
 Maximum Pressure Ramp (psi/sec): 290.0
 20 Step Table:

Step	Total Time(min)	Flow Rate(μl/min)	A (%)	B (%)
0	0.00	200	45.0	55.0
1	10.00	200	45.0	55.0

 Left Compressibility: 50.0
 25 Right Compressibility: 115.0
 Left Dead Volume (μl): 40.0
 Right Dead Volume (μl): 40.0
 Left Stroke Volume (μl): -1.0
 Right Stroke Volume (μl): -1.0
 30 Left Solvent: A2 (water + 2% formic acid)
 Right Solvent: B1 (methanol + 2% formic acid)

CTC PAL Auto sampler Method Properties :

35 Loop Volume1 (μl): 50
 Loop Volume2 (μl): 50
 Injection Volume (μl): 200.000
 Method Description:
 Syringe: 250ul
 40 Analyst LC-Inj
 Air Volume (μl) 0
 Pre Clean with Solvent 1 () 2
 Pre Clean with Solvent 2 () 1
 Pre Clean with Sample () 0
 45 Filling Speed (μl/s) 50
 Filling Strokes () 0
 Inject to LC Vlv1
 Injection Speed (μl/s) 50
 Pre Inject Delay (ms) 500
 50 Post Inject Delay (ms) 500
 Post Clean with Solvent 1 () 3
 Post Clean with Solvent 2 () 2
 Valve Clean with Solvent 1 () 2
 Valve Clean with Solvent 2 () 1
 55

Gas pressure		Auto sampler CTC Analytics HTS Pal (n° 1303)	
N2	4 bar	Peltier rack	10 °C
Air gas	7.5 bar	Loop	50 µL
Air Exhaust gas	4 bar	Solvent 1	H ₂ O + 0.5% HCOOH
		Solvent 2	MeOH
		Oven Agilent G1316A (n°1294)	
		Temperature	Not used
		Valco valve	Column selector
		C2-0000EP V1C1 (n°1289)	C5-0006EMTD (n° 1290)
		Ten ports	6 positions
		2 positions	Not used
		Not used	
Pumps		Source n° 1291	Probe TurbolonSpray n° 1288
Binary pump Agilent 1100 G1312A (n° 1297)	1400 psi		
Flow	0.2 mL/min	Horizontal Position	7 (x axis)
A2= H ₂ O + 2% Formic Acid	45 %	Vertical Position	7 (y axis)
B1= MeOH + 2% Formic Acid	55 %	Capillary exit	1 mm
Isocratic			
		Mass Spectrometer API 4000	
		Device GLP n° 1292	
Binary pump Agilent 1100 G1312A (n° 1296)	Not used		
Flow	-	Column	
A =	-	Precolumn	none
B =	-	Column	SMAR 68-1
Isocratic mode		Thermo Hypercarb 100 x 2.0mm 5µm (ambient temperature)	

Appendix 6
Results obtained with confirmatory transitions

Sample Material	Compound	Fortification Level [mg/L]	Recovery				
			Single Values [%]			Mean [%]	RSD [%]
Drinking water	fosetyl-Al	0.0001	77	75	81	85	12.8
			91	101			
		0.001	78	73	86	80	6.7
			85	79			
			Overall Mean and RSD			83	10.2
	phosphorous acid	0.0001	62	67	86	79	16.6
			89	89			
		0.001	78	77	84	86	11.0
			94	98			
			Overall Mean and RSD			82	13.9
Surface water	fosetyl-Al	0.0001	96	101	106	107	9.4
			122	112			
		0.001	99	100	96	97	2.9
			94	94			
			Overall Mean and RSD			102	8.8
	phosphorous acid	0.0001	116	108	104	109	4.0
			109	108			
		0.001	100	96	93	93	5.6
			88	88			
			Overall Mean and RSD			101	9.5

Appendix 7
Results obtained with substitute LC conditions

5

Drinking water

Calibration curve	Compound	Fortification Level [mg/L]	Recoveries				RSD [%]
			Single Values [%]		Mean [%]		
standards in pure solvent	fosetyl-Al	0.0001	10	10	9	9	9.1
			9	8			
		0.001	18	18	17	18	4.7
			17	19			
			Overall Mean and RSD			14	34.1
	phosphorous acid	0.0001	35	35	37	36	4.6
			38	34			
		0.001	29	28	33	30	6.2
			30	30			
			Overall Mean and RSD			33	10.6
matrix matched standards	fosetyl-Al	0.0001	95	94	85	89	6.3
			88	82			
		0.001	96	97	94	97	4.0
			93	103			
			Overall Mean and RSD			93	6.6
	phosphorous acid	0.0001	93	94	98	96	4.6
			102	91			
		0.001	85	81	95	87	5.9
			86	86			
			Overall Mean and RSD			91	7.2

Surface water

Calibration curve	Compound	Fortification Level [mg/L]	Recoveries				
			Single Values [%]			Mean [%]	RSD [%]
standards in pure solvent	fosetyl-Al	0.0001	26	26	30	30	17.5
			31	39			
		0.001	45	59	71	59	15.7
			60	59			
			Overall Mean and RSD			45	37.1
	phosphorous acid	0.0001	51	63	64	60	9.2
			59	64			
		0.001	69	73	71	70	3.5
			67	68			
			Overall Mean and RSD			65	9.8
matrix matched standards	fosetyl-Al	0.0001	103	102	111	112	10.1
			113	130			
		0.001	98	127	151	126	14.9
			128	126			
			Overall Mean and RSD			119	13.8
	phosphorous acid	0.0001	89	106	109	103	8.2
			101	109			
		0.001	105	110	108	105	3.6
			101	102			
			Overall Mean and RSD			104	6.0

Appendix 8
Flow chart of variant method

Main point : for each set of samples, it is necessary to do a blank reagent where water sample is replaced by H₂O milliQ to be sure that no H₃PO₃ contamination (< 30% LOQ) coming from sample preparation is found.

0.6 g of washed AG 50W-X8 resin
(in disposable Nalgene bottle PP)

+

20.0 g of water sample
(using disposable Pasteur Pipette)

(Add here using a dilutor 200 μ L of the fortifying solution for LOQ recoveries
or 200 μ L of the fortifying solution for 10LOQ recoveries)



Mechanical agitation (10 minutes) at ambient temperature :
Extract A corresponds to Final Extract



HPLC Measurement with Electrospray MS/MS detection

If necessary to dilute the Final Extract due to concentration outside the calibration curve : use final extract of control sample

5 (because matrix matched standards are used for calibration)

Appendix 9

Results obtained with variant method

5

Drinking water

Calibration curve	Compound	Fortification Level [mg/L]	Recoveries					
			Single Values [%]			Mean [%]	RSD [%]	
standards in pure solvent	fosetyl-Al	0.0001	99	97	99	97	2.1	
			98	94				
		0.001	101	98	91	96	3.9	
			95	95				
			Overall Mean and RSD			97	3.0	
	phosphorous acid	0.0001	no result					
		0.001	105	100	101	101	2.0	
			101	100				
			Overall Mean and RSD			101	2.0	
matrix matched standards	fosetyl-Al	0.0001	106	104	106	104	2.0	
			105	101				
		0.001	101	98	92	96	3.5	
			95	96				
			Overall Mean and RSD			100	5.0	
	phosphorous acid	0.0001	no result					
		0.001	98	92	93	94	2.7	
			93	92				
			Overall Mean and RSD			94	2.7	

Surface water

Calibration curve	Compound	Fortification Level [mg/L]	Recoveries				
			Single Values [%]			Mean [%]	RSD [%]
standards in pure solvent	fosetyl-Al	0.0001	103	87	101	97	7.9
			103	90			
		0.001	101	102	98	101	1.7
			102	100			
		Overall Mean and RSD			99	5.7	
	phosphorous acid	0.0001	no result				
		0.001	97	97	94	97	2.1
			99	99			
			Overall Mean and RSD			97	2.1
	matrix matched standards	fosetyl-Al	0.0001	104	88	102	98
104				91			
0.001			101	101	98	100	1.6
			102	99			
		Overall Mean and RSD			99	5.4	
phosphorous acid		0.0001	no result				
		0.001	93	93	90	93	2.2
			95	95			
			Overall Mean and RSD			93	2.2

5 Example 5

This detailed example concerns the analysis of fosetyl-Al and of phosphorous acid using soil samples. This example is a modification M001 to the Analytical Method 00974 for the determination of residues of fosetyl-Al and its metabolite (Phosphorous acid) in soils.

Data Requirement

- EU Council Directive 91/414/EEC amended by Commission Directive 96/46/EC
- European Commission Guidance Document for Generating and Reporting Methods of Analysis in Support of Pre-Registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414, SANCO/3029/99
- European Commission Guidance Document for Residue Analytical Methods, SANCO/825/00 rev.7

10 Summary

The presented residue analytical method modification 00974/M001 was validated for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in soils by LC/MS/MS.

- 15 The residues of fosetyl-Al and its metabolite (phosphorous acid) are extracted from the soil samples by shaking with ammonia buffer solution. This extract is then treated with a cationic resin and a final dilution is done before the analysis by LCMSMS. The residues are quantified by HPLC using an Hypercarb column and detected by tandem mass spectrometry with electrospray ionisation. The quantification was done by an external standardisation using standards prepared in pure solvent.

The validation set included the determination of the detector linearity, the limit of quantification and the accuracy of the method.

- 25 The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at concentrations between 0.25 and 25 µg/L.

The occurrence of matrix effects was monitored.

The conclusion is that :

- 30 - no significant difference is observed when the measurement of fosetyl-Al is established using either standards prepared in solvents, or matrix matched standards ;
- the measurement of phosphorous acid is better (higher mean recoveries and lower RSD found) when it is established using standards prepared in solvents, instead of using matrix matched standards.

- 35 For all these reasons, standards prepared in solvents should be used.

The apparent residues for all control samples were below 20% of the LOQ for each compound, i.e. < 0.01 mg/kg.

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of 20% could be obtained. The LOQ was set at 0.050 mg/kg for each compounds in soils samples.

The accuracy of the method can be assessed on the basis of the determined recovery rates.

- 5 The single recovery rates were in the range of 70 to 81 % for fosetyl-Al and of 80 to 98 % for phosphorous acid. All mean recoveries rates were in the range of 70 to 110 %. The accuracy of the method fulfils the requirements for residue analytical methods which demand that the mean recoveries for each fortification level should be in the range of 70-110%.

- 10 The precision and repeatability of the method can be assessed on the basis of the determined relative standard deviations (RSD) for the mean values of the recovery rates. All RSD were well below 20 %. Thus the precision and repeatability of the method can be considered acceptable.

All results of the method validation are in accordance with the general requirements for residue analytical methods, so this method modification has been validated successfully.

15

1 Introduction

Fosetyl-Al is a fungicide.

- 20 The method modification 00974/M001 presented in this report was validated in order to suppress the steps of derivatization, to change the analysis and detection modes and to decrease the Limit of Quantification (LOQ) of original method 00974 from 0.100 mg/kg to 0.050 mg/kg for fosetyl-Al and phosphorous acid.

Table 29: LOQ and principle of analytical determination

Compound	Fosetyl-Al	Phosphorous acid
Determined as	Fosetyl-Al	Phosphorous acid
Calculated as	Fosetyl-Al	Phosphorous acid
Principle of Determination	LC/MS/MS	LC/MS/MS
LOQ [mg/kg] Soil sample	0.05	0.05

i. defined as the lowest validated fortification level

25

1.1 Citation of the Original Method

Original Method : 00974

Compounds : fosetyl-Al and its metabolite (phosphorous acid)

Reason for Modification : • Suppress the steps of derivatization.

i. defined as the lowest validated fortification level

•Change analysis and detection modes from GC/FPD
to LC/MS/MS.

•Decrease the LOQ from 0.100 mg/kg to 0.050 mg/kg
for fosetyl-Al and phosphorous acid

5

1.2 Physical and Chemical Properties

See example 3.

2 Experimental Section

10

2.1 Materials

2.1.1 Apparatus

Standard laboratory glass consumable should be cleaned with only detergents containing no phosphate and rinsed with water and acetone.

To avoid any contamination, the use of disposable laboratory consumable is strongly advised.

15

• Balances :

➤ accuracy $\pm$ 0.1 mg (analytical standards) (e.g. Mettler AT261 range)

➤ accuracy $\pm$ 0.1 g (samples) (e.g. Mettler PM2000)

- Dilutor (e.g. Hamilton MicroLab 1000 plus)
- Rotary shaker (e.g. Heidolph REAX 2)
- Centrifuge (e.g. Hermle Z513K)

20

(ex Hettich EBA12)

- HPLC (e.g. Binary Pump Agilent 1100)
- Autosampler (e.g. CTC Analytics HTC PAL)
- Triple Quadrupole HPLC-MS/MS Mass Spectrometer (e.g. Sciex Instruments, API 4000 System)
- Column (e.g. Hypercarb, 100 x 2.0 mm, 5 μ m)

25

3.1.2 Reagents and Supplies

- Acetone (e.g. Pestipur® SDS)
- Methanol (e.g. Pestipur® SDS)
- Formic acid (e.g. Analytical reagent ® Merck)
- Ammonium hydrogen carbonate (e.g. Normapur® Merck)
- ammonia solution 32% (e.g. Rectapur ® Merck)
- Cationic resin (AG 50W-X8, 20-50 Meshs, hydrogen form) (e.g. Bio Rad)
- GF/A filters (125 mm), (e.g. Whatman)
- Polypro bottles (1000 mL, wide opening) (e.g. Nalgen)
- Polypro bottles (250 mL, wide opening) (e.g. Nalgen)
- Polypro bottles (125 mL, wide opening) (e.g. Nalgen)
- Polypro bottles (60 mL, wide opening) (e.g. Nalgen)
- Polypro funnels (67mm) (e.g. Marylands Plastics)
- Conical centrifuge tube (15 mL) (e.g. Merck)

30

35

40

- Solvent for dilution : Water with 0.5 % HCOOH
- Mobile phase solvent : Water with 2 % HCOOH

Methanol with 2 % HCOOH

- ammonia buffer solution : pour 500 mL of water in a 1000 mL volumetric flask, add
- 5 20 g of ammonium hydrogen carbonate and mix with a magnetic stirrer until complete solubilisation. Add 15 mL of ammonia solution 32% and complete to 1000 mL with water.

2.1.3 Reference Item

Only sufficiently characterised and certified item was used as reference item.

- 10 The reference item was made available by Bayer CropScience GmbH produkt Analytik, G864, Industriepark Höchst, D-65926 Frankfurt-am-Main, Germany.

Table 30: Reference item data

Name of Substance	Batch Number	Content [%]	Date of Expiry
fosetyl-Al	12/1080	97.6	February 20, 2006
phosphorous acid	04911DN	96.2	March 02, 2008

15 2.1.4 Standard Solutions

Stock and standard solutions were stored protected from light in a refrigerator at around 5°C.

Stock solutions (1000 mg/L)

- Into a 100 mL amber screw-cap flask, weigh accurately between 20 and 50 mg of reference item. Using a burette, add a volume of water to obtain a stock solution of exactly 1000 mg/L. Mix
- 20 thoroughly until complete dissolution using a magnetic stirrer. Two separate stock solutions must be prepared for each compound. After comparison of these two stock solutions, they are mixed.

Mixture solutions

- Pipette 5 mL of each stock solution using a class "A+" pipette. Pour into a class "A" 50 mL
- 25 volumetric flask. Adjust volume with water, cap and mix by shaking (100 mg/L of each compound). Two different mixture solutions are prepared.

Fortifying standard solutions

- By dilution in water of one of the mixture solution, which is also used as fortifying solution for recoveries at 10LOQ level (100 mg/L of each compound), prepare the fortifying standard
- 30 solution used for recoveries at LOQ level (10 mg/L of each compound).

Intermediate standard solution

By serial dilutions of the other mixture solution, prepare the intermediate standard solution at 1.0 mg/L of each compound using water.

Before use, the intermediate solution and the fortifying standard solution used for recoveries at LOQ level are compared to validate their preparation.

Standard solutions in solvent used for calibration

To obtain the standard solutions used for calibration, dilute extemporaneously using a dilutor (or in a different way) and water with 0.5% formic acid, the intermediate standard solution to obtain the following concentrations: 0.25, 0.4, 0.5, 1, 2.5, 5, 10 and 25 µg/L.

Matrix matched standard solutions

The occurrence of matrix effects was monitored and the measurement of both compounds is established using matrix matched standards in both sample materials.

From the intermediate standard solution, the dilutions are the same as preparation of standard solutions in solvent, except the dilution mixture which is the final extract of a control sample.

Remarks :

- 20 to 25 mL of final extract are necessary to make all dilutions.
- to avoid the use of standard laboratory glass consumable, which could bring some H3PO3 contamination, the dilution of the control sample extract B is done by weighing. Using disposable Pasteur pipette weigh 3.0 g of extract B into a 60 mL polypropylene bottle. Using another disposable Pasteur pipette, make-up to 30.0 g with acidified water with formic acid 0.5 % : this is the control sample final extract used as dilution mixture to prepare matrix matched standard solutions.

2.1.5 Stability of the standard solutions

The stock solutions, stored protected from light in a refrigerator at around 5°C, were found to be stable for 9 months and a half.

2.2 Residue Analytical Methodology

Some modifications compared to the original analytical method were introduced.

The method modification 00974/M001 presented in this report was validated in order to suppress the steps of derivatization, to change the analysis and detection modes and to decrease the Limit of Quantification (LOQ) of original method 00974 from 0.100 mg/kg to 0.050 mg/kg for each compound.

- The Limit of Quantification was decreased from 0.100 mg/kg to 0.050 mg/kg for fosetyl-Al and phosphorous acid.
- The derivatization step was suppressed.
- The quantification was carried out by LC/MS/MS instead of GC/FPD.

All modifications were included in the description below.

A flow chart of the method is given in Appendix 7.

Note : during analysis, for each sample set, it is necessary to do a blank reagent without soil sample to be sure that no phosphorous acid contamination (< 30% LOQ) coming from sample preparation is found.

5

Remark : standard laboratory glass consumable should be cleaned with only detergents containing no phosphate and rinsed with water and acetone.

To avoid any contamination, the use of disposable laboratory consumable is strongly advised.

- 10 For recovery experiments, samples are fortified by adding the appropriate standard solution to the sample material after weighing and before shaking.

Preparation of the cationic resin :

1. Weigh about 25 g of AG 50W-X8 resin into a 1000 mL polypropylene bottle.
- 15 2. Add about 500 mL of water.
3. Shake using a rotary shaker for about 10 minutes.
4. Discard the supernatant water.
5. Do steps 2 to 4 a second time.
6. Do steps 2 to 4 a third time.
- 20 7. Filtrate residual water and resin through two GF/A filters put in a polypropylene funnel and previously rinsed with water.

Remark: resin is prepared either just before use or in advance, stored at ambient temperature and rehydrated just before use.

- 25 Caution: phosphorous acid interferences could be observed if the resin is not washed as described above.

Samples preparation :

1. Weigh 20.0 g of homogeneous sample material into a 125 mL polypropylene bottle.
- Note: weight of the sample is used for residue calculation, as variable Weight_{Sample}.
- 30 2. Add 30 mL of ammonia buffer solution.
 3. Shake the sample using a rotary shaker for 30 minutes at ambient temperature.
 4. Centrifuge the extract (3600 rpm – 5°C) for approx. 5 minutes.
 5. Pour about 100 g of acidified water with formic acid 0.5 % into a 250 mL polypropylene bottle previously weighted.
 - 35 6. Add the supernatant into the 250 mL polypropylene bottle.
 7. Add 30 mL of ammonia buffer solution on the bottom.
 8. Shake the sample using a rotary shaker for 30 minutes.
 9. Centrifuge the extract (3600 rpm – 5°C) for approx. 5 minutes.

10. Pour the supernatant into the 250 mL polypropylene bottle.

11. Using disposable Pasteur pipette, make-up to 200 g with acidified water with formic acid 0.5 %. This is the Extract A (for information, the pH is around 6.5).

Note: weight of extract A is used for residue calculation, as variable $Weight_{Extract\ A}$.

5 12. Weigh 3.0 g of AG 50W-X8 resin previously washed into a conical centrifuge tube.

13. Using a disposable glass pipette, transfer an aliquot of 5 mL of Extract A into the centrifuge tube.

14. Shake the sample using a rotary shaker for 10 minutes.

10 15. Centrifuge the sample (6000 rpm – ambient) for approx. 5 minutes : the supernatant obtained corresponds to Extract B (for information, the pH is around 2.5).

Note: this step of resin treatment is necessary to obtain a narrow H3PO3 peak shape.

16. Dilute using a dilutor (or in a different way) ten times the extract B using acidified water with formic acid 0.5 %. This is the Final Extract (for information, the pH is around 2.5).

17. Proceed to LC/MS/MS measurement, Chapter 3.3.

15 18. If necessary to dilute the Final Extract due to a concentration outside the calibration curve : use acidified water with formic acid 0.5 %.

2.3 Analysis and Instrument Conditions

20 The final extracts are injected into a high performance liquid chromatograph and detected by tandem mass spectrometry with electrospray ionisation.

The quantification is carried out by external standardisation using standards prepared in solvent. Exemplary LC/MS/MS conditions that were used in the course of this method validation are listed in chapters 3.3.1 and 3.3.2. These conditions are given as a guidance and may have to be adapted for other HPLC-MS/MS systems.

25

2.3.1 HPLC Conditions

Instrument: Binary pump Agilent 1100

Autosampler: CTC Analytics HTS PAL

Column: Hypercarb, 100 x 2.0 mm, 5 μ m

30 Precolumn: none

Injection Volume: 50 μ L

Column temperature: ambient (about 25°C)

Mobile Phase: Isocratic mode: 55 / 45 (v/v) Methanol + 2% formic acid / water + 2% formic acid

35 Flow (Column): 200 μ L/min

Retention Times: about 3.1 min for phosphorous acid and 4.2 min for fosetyl-Al.

Remarks :

- It is necessary to wait about 2 hours the stabilisation of the HPLC system before injecting. During a samples set, a light drift of retention time of both compounds can be observed.
- Hypercarb precolumn must not be used.
- Depending of the phase batch of Hypercarb column, the percentage of formic acid in the mobile phase could be adapted to improve peaks shape (from 0.5% to 2%).
- At the contrary, do not increase the HCOOH rate in the injection solvent. The use of water at 0.5% formic acid as injection solvent is necessary to keep a narrow H₃PO₃ peak shape.

2.3.2 MS/MS Conditions

The experiments were performed on a triple-quadrupole mass spectrometer system, fitted with an electrospray interface operated in the negative ion mode under MRM (multiple reaction monitoring) conditions.

For instance:

Detector: Triple Quadrupole HPLC-MS/MS Mass Spectrometer,
e.g. Sciex Instruments, API 4000 System

Source: TIS (Turbo Ion Spray)

Temperature: 650°C

Scan Type: MRM-Mode (Multiple Reaction Monitoring Mode)

Polarity: Negative ion mode

Gas Flows: Nebulization Gas Air (GS1): 40

Turbo Gas Air (GS2): 60

Curtain Gas N₂ (CUR): 15

Collision Gas N₂ (CAD): 6

Collision Energy:

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (V)
Fosetyl-Al	109	81	200	-16
Phosphorous acid	81	79	400	-22

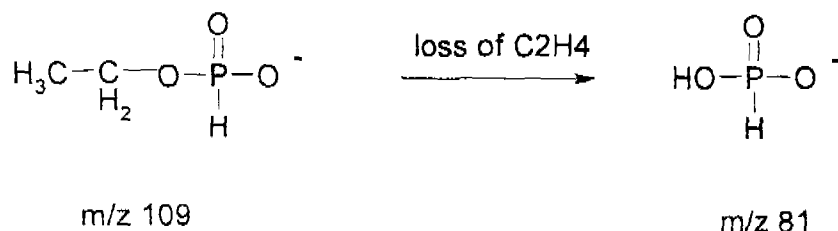
Table 31: Mass spectrometer scan parameters for the quantifier ions used. The detailed instrument settings used are given in chapters 3.3.1 and 3.3.2. Varying instrument systems or instrument parameters may result in different ion transitions and different relative intensities.

Note: Some mass spectrometer conditions are instrument specific. The spectrometer conditions should be optimised by a competent operator prior to analysis.

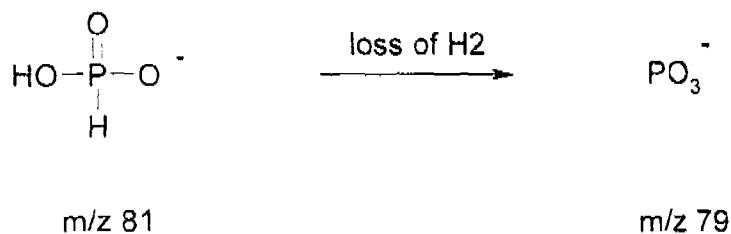
- 5 Details on MS/MS and LC conditions are given in Appendix 8.

The fragmentation pathways for the quantifier ions for fosetyl-Al and phosphorous acid are shown in Figure 1 and in Figure 2.

- 10 Figure 1: Proposed fragmentation pathway for fosetyl-Al



- Figure 2: Proposed fragmentation pathway for phosphorous acid



15

2.3.3 Confirmatory transitions

To confirm or exclude some interference or pollution in samples, the following transitions can be used in the same conditions described above :

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (V)
fosetyl-Al	109	63	300	-38
phosphorous acid	81	63	400	-38

- 20 The fragmentation pathways for the confirmatory transitions for fosetyl-Al and phosphorous acid are shown in Figure 3 and in Figure 4.

Figure 3: Proposed fragmentation pathway for fosetyl-Al

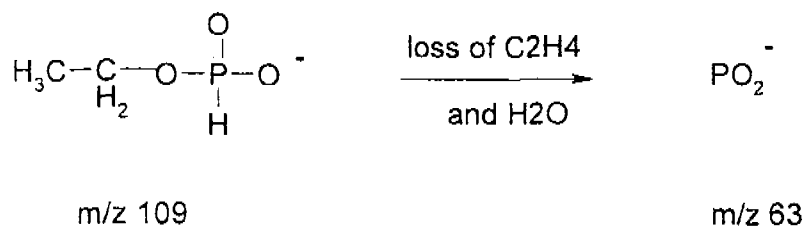
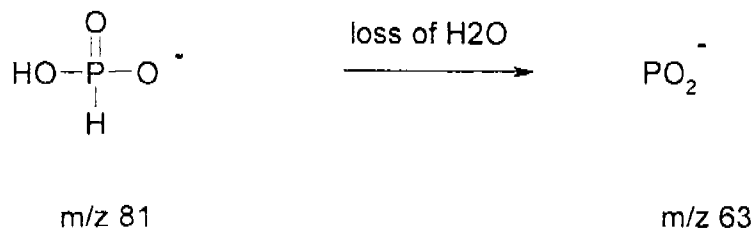


Figure 4: Proposed fragmentation pathway for phosphorous acid



Note : all recovery samples were also analysed using confirmatory transitions. The results are given in Appendix 9.

2.4 Linearity of the Detector

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at concentrations between 0.25 and 25 µg/L.

Table 32: Standard concentrations prepared for the determination of detector linearity. The concentration corresponding to the LOQ is is printed in bold figures.

HPLC-MS/MS	Standard Concentrations [µg/L]							
fosetyl-Al	0.25	0.4	0.5	1	2.5	5	10	25
phosphorous acid	0.25	0.4	0.5	1	2.5	5	10	25

2.5 Storage Stability of Extracts

The stability of sample extracts containing fosetyl-Al and phosphorous acid was not determined in this study.

2.6 Calculation

2.6.1 Calculation of Residues

Evaluation in this case is performed according to the external standardisation using standards prepared in solvent.

- 5 During the analysis of each set of samples, the 8 standard solutions mentioned in Table 4 are injected once. Standards should be interspersed with samples to compensate for any minor change in instrument response.

For each compound, the peak area is plotted versus the concentration in order to establish a calibration curve obtained by linear regression weighting $1/x$ with least squares method.

- 10 The corresponding model to determine the concentration in final extracts is calculated using the Analyst Software (Version 1.4).

Each final extract is injected once using the same conditions as previously described for the standard solutions.

- 15 Using the predicting mathematical model previously established, the final concentration in $\mu\text{g/L}$ of each compound is determined for each injection.

For each compound, the amount of residue R, expressed in mg/kg is calculated, using the following formula :

$$R = \frac{C \times \text{Weight}_{\text{Extract A}} \times \text{Density}^{-1} \times D}{1000 \times \text{Weight}_{\text{Sample}}}$$

where:

- 20 R : Determined amount of residue of fosetyl-Al or phosphorous acid in soil sample in mg/kg
- C : Concentration of fosetyl-Al or phosphorous acid found in the analysed extract in $\mu\text{g/L}$
- Weight_{Extract A} : Weight of the extract A in g, here 200 g
- 25 Density⁻¹ : 1 mL/g
- D : Dilution factor to obtain the Final Extract, here 10
- Weight_{Sample} : Sample weight of analytical sample in g, here 20 g

Remarks :

- 30 • The density of all solutions used during the sample preparation (ammonia buffer solution and H_2O at 0.5% HCOOH) is considered equal to 1 independently to room temperature. This allows to convert the extract weight into extract volume.
- If it is necessary to express phosphorous acid in fosetyl-Al equivalent, the ratio of the molecular weight must be used :

$$\text{Ratio} = \frac{354.1 \text{ g/mol}}{(82.0 \times 3) \text{ g/mol}} = 1.44$$

because 1 mole fosetyl-Al (354.1g) gives 3 moles of phosphorous acid.

2.6.2 Calculation of Recovery Rates

- 5 The concentration of each compound in µg/L is determined for the recovery sample according to 2.6.1.

The percent recovery rate is then calculated as follows :

$$\text{Rec} = \frac{C \times 100}{A}$$

where: Rec : Recovered amount found in fortified sample in %

C : Concentration of fosetyl-Al or phosphorous acid found in the analysed extract in µg/L

A : Fortified amount of fosetyl-Al or phosphorous acid in µg/L

10 2.6.3 Calculation of Relative Standard Deviation (RSD)

The RSD is calculated as follows :

$$\text{RSD (\%)} = \text{S.D.} / \text{Mean Recovery} \times 100 \%$$

$$\text{S.D.} = \left[\frac{\sum (R_i - R_m)^2}{n - 1} \right]^{1/2}$$

R_i : recovery

R_m : mean recovery

n : number of recoveries

15 3 Results and Discussion

3.1 Specificity and Selectivity

The method allows the determination of fosetyl-Al and its metabolite (phosphorous acid) in soil samples.

- 20 The specificity of the method resulted from the HPLC separation in combination with the very selective MS/MS detection.

3.2 Apparent residues in Control Samples

The method was validated using the two German soils Höfchen and Laacher Hof.

- 25 Two different soils were used in order to assess a possible influence of different soil characteristics. The soil samples were classified according to DIN and/or USDA specifications. Soil characteristics of the used soils are summarised in Table 33.

Complete soil characterisation is reported in the Appendix 10.

Table 33: Soil Types

Soil	Texture of Soil	Organic Matter [%]
Höfchen	silt loam (USDA)	1.58
Laacher Hof	sandy loam (USDA)	2.06

A residue level estimation in control samples was performed. The results are listed in Table 34. The apparent residues for all control samples were below 20% of the LOQ for each compound, i.e. < 0.01 mg/kg.

Table 34: Apparent residues in untreated control samples for fosetyl-Al and phosphorous acid

Sample Material	LOQ [mg/kg]		Apparent residues [mg/kg]	
	fosetyl-Al	phospho- rous acid	fosetyl-Al	phospho- rous acid
Höfchen	0.05	0.05	< 10% LOQ	< 20% LOQ
Laacher Hof	0.05	0.05	< 10% LOQ	< 10% LOQ

3.3 Linearity of the Detectors and Matrix Effects

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at concentrations between 0.25 and 25 µg/L.

Experimental details can be found in Chapter 2.4.

In each chromatogram, the measured peak area of fosetyl-Al or phosphorous acid is plotted versus the corresponding concentration of respectively fosetyl-Al or phosphorous acid contained in each standard solution, in order to obtain calibration curve of the form :

$y = ax + b$ (1/x weighting)

where : y = peak area,
 x = concentration in injected standard solution

The results of the determination of detector response for LC/MS/MS are summarised inTable 35.

Table 35: Summary of the determination of detector linearity for LC/MS/MS.

Detection	Parameter	fosetyl-Al	phosphorous acid
LC/MS/MS	Linear range [µg/L]	0.25 - 25	0.25 - 25
	No. of concentrations	7 or 8	7 or 8
	No. of injections	1	1
	Model	1/x weighted linear regression	1/x weighted linear regression
	Correlation coefficient (R) for standards prepared in solvent	0.9999	≥ 0.9996
	Correlation coefficient (R) for matrix matched standards	≥ 0.9997	0.9999

An excellent linear correlation between the injected amount of the analytes and the detector responses of LC/MS/MS was observed for standards in the range of 0.25 to 25 µg/L for both compounds, using either standards prepared in solvent or matrix matched standards.

The occurrence of matrix effects was monitored.
The results are shown in Table 36 and Table 37.

Table 36: Matrix effect evaluation for fosetyl-Al FL : Fortification Level

Sample Material	FL [mg/kg]	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
	0.05	5	72	2.7	81	3.8

Höfchen	0.05	5	72	2.7	81	3.8
	0.5	5	78	1.1	78	3.2
Laacher Hof	0.05	5	71	2.3	67	5.7
	0.5	5	80	1.4	76	5.8

Table 37: Matrix effect evaluation for Phosphorous acid FL : Fortification Level

Sample Material	FL [mg/kg]	Number of Values (n)	Measurement using			
			Standards in pure solvent		Matrix matched standards	
			Mean [%]	RSD [%]	Mean [%]	RSD [%]
Höfchen	0.05	5	91	3.2	76	10.0
	0.5	5	85	2.3	77	3.9
Laacher Hof	0.05	5	84	9.0	72	14.4
	0.5	5	84	2.0	78	3.1

- 5 The results presented in the two previous Tables 36 and 37 show that :
- no significant difference is observed when the measurement of fosetyl-Al is established using either standards prepared in solvents, or matrix matched standards.
 - the measurement of phosphorous acid is better (higher mean recoveries and lower RSD found) when it is established using standards prepared in solvents, instead of using matrix matched standards.
- 10 For all these reasons, we advice to use standards prepared in solvents.

3.4 Limit of Quantification and Recovery Experiments

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq$ 20% could be obtained. The LOQ was set at 0.050 mg/kg for each compounds in soils samples.

To validate the method for these matrices, control samples were fortified with a defined amount of fosetyl-Al and phosphorous acid prior to analysis.

20

3.5 Recovery Rates

The detailed recovery results obtained are listed in Tables 38 and 39.

All results given in Tables 38 and 39 have been obtained by using standards prepared in solvents and the quantifier ion (see 3.3.2). The results obtained with the confirmatory transition are given in Appendix 9.

5

Table 38: Recovery rates obtained for fosetyl-Al, RSD: relative standard deviation

[illegible]

Table 39: Recovery rates obtained for Phosphorous acid, RSD: relative standard deviation

[illegible]

The obtained recovery rates are summarised below in Table 40.
In total 20 recovery rates were determined for each compound.

5 Table 40: Summary of the recovery data for the determination of accuracy and repeatability;
RSD: relative standard deviation

	Parameter	Fosetyl-Al	Phosphorous acid	Number of values n
Accuracy	Single recoveries [%]	70 - 81	80 - 98	20
	Mean recoveries per fortification level [%]	72 - 79	84 - 88	10
	Mean recoveries per kind of soils [%]	75 - 76	84 - 88	10
	Overall mean [%]	75	86	20
Repeatability	RSD per fortification level [%]	1.5 - 2.4	2.0 - 7.3	10
	RSD per kind of soils [%]	4.7 - 6.0	4.6 - 6.2	10
	Overall RSD [%]	5.3	5.6	20

The previous table shows that .

The single recovery rates were in the range of 70 to 81 % for fosetyl-Al and of 80 to 98 % for phosphorous acid. All mean recoveries rates were in the range of 70 to 110 %.
All RSD were well below 20 %.

4 Evaluation and Discussion

The presented residue analytical method modification 00974/M001 was validated for the determination of residues of fosetyl-Al and its metabolite (phosphorous acid) in soils by LC/MS/MS.

The residues of fosetyl-Al and its metabolite (phosphorous acid) are extracted from the soil samples by shaking with ammonia buffer solution. This extract is then treated with a cationic resin and a final dilution is done before the analysis by LCMSMS. The residues are quantified by HPLC using an Hypercarb column and detected by tandem mass spectrometry with electrospray ionisation. The quantification was done by an external standardisation using standards prepared in pure solvent.

The validation set included the determination of the detector linearity, the limit of quantification and the accuracy of the method.

The linearity of the detector used was tested for fosetyl-Al and phosphorous acid using standards in solvent and matrix matched standards.

The linearity was tested by injecting standards of fosetyl-Al and phosphorous acid at concentrations between 0.25 and 25 µg/L. The detector response was linear in these ranges.

5

The occurrence of matrix effects was monitored

- no significant difference is observed when the measurement of fosetyl-Al is established using either standards prepared in solvents, or matrix matched standards ;

10 - the measurement of phosphorous acid is better (higher mean recoveries and lower RSD found) when it is established using standards prepared in solvents, instead of using matrix matched standards.

For all these reasons, we advice to use standards prepared in solvents.

15 The apparent residues for all control samples were below 20% of the LOQ for each compound, i.e. < 0.01 mg/kg.

The limit of quantification (LOQ) was defined as the lowest fortification level where a mean recovery within the range of 70 to 110% and an RSD of $\leq$ 20% could be obtained. The LOQ was set at 0.050 mg/kg for each compounds in soils samples.

20 The accuracy of the method can be assessed on the basis of the determined recovery rates. The single recovery rates were in the range of 70 to 81 % for fosetyl-Al and of 80 to 98 % for phosphorous acid. All mean recoveries rates were in the range of 70 to 110 %. The accuracy of the method fulfils the requirements for residue analytical methods which demand that the mean recoveries for each fortification level should be in the range of 70-110%.

25 The precision and repeatability of the method can be assessed on the basis of the determined relative standard deviations (RSD) for the mean values of the recovery rates. All RSD were well below 20 %. Thus the precision and repeatability of the method can be considered acceptable.

30 All results of the method validation are in accordance with the general requirements for residue analytical methods, so this method modification has been validated successfully.

Note : during analysis, for each sample set, it is necessary to do a blank reagent without soil sample to be sure that no phosphorous acid contamination (< 30% LOQ) coming from sample preparation is found.

35 Remarks :

- standard laboratory glass consumable should be cleaned with only detergents containing no phosphate and rinsed with water and acetone.

To avoid any contamination, the use of disposable laboratory consumable is strongly advised.

• Before start of validation / measurements equipment / chemicals should be tested for any residues of phosphorous acid.

• If some contamination is observed, see if the use of special HPLC vials (e.g. polypropylene vials, Agilent, art. 5182-0567) can be helpful to reduce the background for phosphorous acid.

Appendix 7

Flow Diagram of Residue Method 00974/M001

Soil sample preparation procedure

Main point : for each set of samples, it is necessary to do a blank reagent without soil sample to be sure that no H_3PO_3 contamination (< 30% LOQ) coming from sample preparation is found (that is why disposable consumables are used)

20.0 g of soil sample into 125 mL polypropylene bottle
(Add here 100 μ L of the fortifying solution for LOQ recoveries
or 100 μ L of the fortifying solution for 10LOQ recoveries)

+
30 mL of ammonia buffer solution

Mechanical agitation (~30 minutes) at ambient temperature

Centrifugation (~5 minutes at ~3600rpm at ~5°C)

Bottom

+ 30 ml of ammonia buffer solution

Mechanical agitation (~30 minutes) at ambient temperature

Centrifugation
(~5 minutes at ~3600rpm at ~5°C)

Put the floating into a weighted disposable 250 mL polypropylene bottle where about 100 g of acidified water at 0.5% formic acid were already poured

Floating

Make-up to 200 g with H_2O at 0.5% $HCOOH$,
using Pasteur pipette : **Extract A**

Weigh 3 g of washed AG 50W-X8 resin into a conical centrifuge tube

Transfer about 5 mL of Extract A into this centrifuge tube, using disposable pipette

Mechanical agitation for ~10 minutes at ambient temperature

Then centrifuge (~5 minutes at ~6000 rpm – ambient):
the supernatant obtained corresponds to **Extract B**

Dilute 10 times the extract B in H_2O + 0.5% $HCOOH$ = **Final Extract for LCMSMS**

If necessary to dilute the Final Extract due to concentration outside the calibration curve : use H_2O + 0.5% $HCOOH$

Remark : ~ means 'around'.

Appendix 8

Details on LC-MS/MS conditions

Comment:

5 Synchronization Mode: LC Sync
 Auto-Equilibration: Off
 Acquisition Duration: 10min1sec
 Number Of Scans: 455
 Periods In File: 1
 10 Acquisition Module: Acquisition Method
 Software version: Analyst 1.4

MS Method Properties:

15 Period 1:

 Scans in Period: 455
 Relative Start Time: 0.00 msec
 Experiments in Period: 1

20

Period 1 Experiment 1:

 Scan Type: MRM (MRM)
 Polarity: Negative
 25 Scan Mode: N/A
 Ion Source: Turbo Spray
 Resolution Q1: Unit
 Resolution Q3: Unit
 Intensity Thres.: 0.00 cps
 30 Settling Time: 0.0000 msec
 MR Pause: 5.0070 msec
 MCA: No
 Step Size: 0.00 amu

35

Quantifier transitions:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
80.90	78.90	400.00	DP	-55.00	-55.00
(phosphorous acid)			CE	-22.00	-22.00
			CXP	-5.00	-5.00

40

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
108.90	80.90	200.00	DP	-45.00	-45.00
(fosetyl-Al)			CE	-16.00	-16.00
			CXP	-1.00	-1.00

45

Confirmatory transitions:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
80.90	62.90	400.00	DP	-55.00	-55.00
(phosphorous acid)			CE	-38.00	-38.00
			CXP	-1.00	-1.00

50

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	Start	Stop
108.90	62.90	300.00	DP	-45.00	-45.00
(fosetyl-Al)			CE	-38.00	-38.00
			CXP	-5.00	-5.00

55

Parameter Table (Period 1 Experiment 1):

CAD: 6.00
 CUR: 15.00
 GS1: 40.00
 GS2: 60.00
 5 TEM: 650.00
 ihe: ON
 IS: -4500.00
 EP: -10.00

10 Agilent 1100 LC Pump Method Properties :

Pump Model: Agilent 1100 LC Binary Pump
 Minimum Pressure (psi): 0.0
 Maximum Pressure (psi): 5801.0
 15 Dead Volume (µl): 40.0
 Maximum Flow Ramp (ml/min²): 100.0
 Maximum Pressure Ramp (psi/sec): 290.0
 Step Table:

Step	Total Time(min)	Flow Rate(µl/min)	A (%)	B (%)
20 0	0.00	200	55.0	45.0
1	10.00	200	55.0	45.0

 Left Compressibility: 50.0
 Right Compressibility: 115.0
 Left Dead Volume (µl): 40.0
 25 Right Dead Volume (µl): 40.0
 Left Stroke Volume (µl): -1.0
 Right Stroke Volume (µl): -1.0
 Left Solvent: A1 (methanol + 2% formic acid)
 Right Solvent: B2 (water + 2% formic acid)

30 CTC PAL Autosampler Method Properties :

Loop Volume1 (µl): 50
 Loop Volume2 (µl): 100
 35 Injection Volume (µl): 100.000
 Method Description:
 Syringe: 250ul
 Analyst LC-Inj
 Air Volume (µl) 0
 40 Pre Clean with Solvent 1 () 2
 Pre Clean with Solvent 2 () 1
 Pre Clean with Sample () 0
 Filling Speed (µl/s) 50
 Filling Strokes () 0
 45 Inject to LC Vlv1
 Injection Speed (µl/s) 50
 Pre Inject Delay (ms) 500
 Post Inject Delay (ms) 500
 Post Clean with Solvent 1 () 3
 50 Post Clean with Solvent 2 () 2
 Valve Clean with Solvent 1 () 2
 Valve Clean with Solvent 2 () 1

55

Gas pressure		Autosampler CTC Analytics HTS Pal (GLP n° 1303)	
N2	4 bar	Peltier rack	10 °C
Air GS1/GS2 gas	7 bar	Wash solvent 1 : H2O + 0.5% HCOOH	
Air Exhaust gas	4 bar	Wash solvent 2 : MeOH	
Pumps			
Analytical LC pump :			
Binary 1 pump Agilent 1100 (GLP n° 1296):			
A1 = Methanol + 2% Formic Acid		Valco valve used	Column selector used
B2 = H ₂ O + 2% Formic Acid		GLP n° 1289	GLP n° 1356
		Source GLP n° 1291	
			Probe TurbolonSpray
Pump for making-up solvent :			GLP n° 1288
		Horizontal Position	7 (x axis)
		Vertical Position	7 (y axis)
		Capillary exit	1 mm
		Mass Spectrometer API 4000	
		Device GLP n°	1292
Binary 2 pump Agilent 1100 (GLP n° 1297):			
A2 = H ₂ O + 2% Formic Acid		Column :	at room temperature
B2 = Acetonitrile		Precolumn : none	
		Column : SMAR 68-1	
		Thermo Hypercarb 100 x 2.0mm 5µm	

Appendix 9
Results obtained with confirmatory transitions

All results in Tables 41 and 42 have been obtained by using standards prepared in solvents.

Table 41: Recovery rates obtained for Fosetyl-Al. RSD: relative standard deviation

Fortification [mg/kg]	Soil	Single Values [%]					Mean [%]	RSD [%]
0.05	Höfchen	77	73	72	76	74	74	2.8
0.05	Laacher Hof	72	72	76	76	74	74	2.7
mean of all 0.05 mg/kg single values							74	2.6
0.5	Höfchen	79	79	80	76	78	78	1.9
0.5	Laacher Hof	80	80	79	79	81	80	1.0
mean of all 0.5 mg/kg single values							79	1.7
mean of all Höfchen samples							76	3.6
mean of all Laacher Hof samples							77	4.4
overall mean							77	3.9

5

Table 42: Recovery rates obtained for Phosphorous acid, RSD: relative standard deviation

Fortification [mg/kg]	Soil	Single Values [%]					Mean [%]	RSD [%]
0.05	Höfchen	92	87	85	87	85	87	3.3
0.05	Laacher Hof	87	75	78	73	79	78	6.8
mean of all 0.05 mg/kg single values							83	7.4
0.5	Höfchen	86	85	85	81	84	84	2.3
0.5	Laacher Hof	83	83	83	82	86	83	1.8
mean of all 0.5 mg/kg single values							84	2.0
mean of all Höfchen samples							86	3.3
mean of all Laacher Hof samples							81	5.6
overall mean							83	5.3

All results obtained above with confirmatory transition comply with European requirements.

Appendix 10
Soil characterisation

Soil Höfchen

5

Soil Höfchen, plot 4011:	0 – 30 cm soil layer
pH (in CaCl ₂ solution)	6.7
pH (in H ₂ O)	7.4
Organic Carbon [%]	0.92
Organic Matter [%] *	1.58
Cation Exchange Capacity [meq / 100 g dry soil]	12.4
max. Water Holding Capacity [g / 100 g dry soil]	39.4
Textural Description according to USDA	
Fraction [%]	
Clay (<0.002 mm)	19.4
Silt (0.002-0.050 mm)	76.3
Sand (0.050-2.000 mm)	4.3
Soil type: silt loam	

* Organic matter = Organic carbon x 1.72

Soil Laacher Hof

Soil Laacher Hof, plot 712/718:	0 – 30 cm soil layer
pH (in CaCl ₂ solution)	6.8
pH (in H ₂ O)	7.4
Organic Carbon [%]	1.20
Organic Matter [%] *	2.06
Cation Exchange Capacity [meq / 100 g dry soil]	9.8
max. Water Holding Capacity [g / 100 g dry soil]	37.9
Textural Description according to USDA	
Fraction [%]	
Clay (<0.002 mm)	12.0
Silt (0.002-0.050 mm)	18.3
Sand (0.050-2.000 mm)	69.7
Soil type: sandy loam	

* Organic matter = Organic carbon x 1.72

CLAIMS

1. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.00005 mg/kg of a sample, comprising the following steps:
- preparation of the sample ;
 - optional dilution of the sample prepared ;
 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).
2. Method according to Claim 1, comprising a step consisting of dilution of the sample prepared.
3. Method according to either of Claims 1 and 2, for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.000005 mg/kg of sample.
4. Method according to any one of Claims 1 to 3, for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.0000005 mg/kg of sample.
5. Method according to any one of Claims 1 to 4, for which the sample is chosen from plant tissues ; water ; soils ; animal products or tissues ; air ; agrofood products, and human body fluids such as blood and urine.
6. Method according to any one of Claims 1 to 5, for which the sample is chosen from a plant matrix with a high water content, a plant matrix with an acidic pH, a dry plant matrix, a fatty plant matrix ; mineral water, underground water, mains water or surface water ; milk, eggs, liver, kidneys, fats, muscle ; converted agrofood products ; human body fluids such as blood and urine.
7. Method according to any one of Claims 1 to 6, for which the preparation is chosen from an extraction for plant tissues ; soils ; animal products or tissues and converted agrofood products ; optional concentration for water and trapping for air.
8. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.005 mg/kg of a water sample, comprising the following steps:
- preparation of the water sample ;
 - optional dilution of the sample prepared ;
 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

9. Method of analysis according to Claim 8, for the analysis of amounts of less than or equal to 0.00005 mg/kg.

5 10. Method of analysis according to either of Claims 8 and 9, for the analysis of amounts of less than or equal to 0.0000005 mg/kg.

11. Method according to any one of Claims 8 to 10, for which the sample is chosen from mineral water, underground water, mains water or surface water.

10 12. Method according to any one of Claims 8 to 11, for which the preparation is an optional concentration of the water.

13. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 1 mg/kg of a plant tissue sample, comprising the following steps:

- 15
- preparation of the plant tissue sample ;
 - optional dilution of the sample prepared ;
 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

20 14. Method of analysis according to Claim 13, for the analysis of amounts of less than or equal to 0.01 mg/kg.

25 15. Method of analysis according to either of Claims 13 and 14, for the analysis of amounts of less than or equal to 0.001 mg/kg.

16. Method according to any one of Claims 13 to 15, for which the sample is chosen from a plant matrix with a high water content, a plant matrix with an acidic pH, a dry plant matrix and a fatty plant matrix.

30 17. Method according to any one of Claims 13 to 16, for which the preparation is an extraction of the plant tissue.

18. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 5 mg/kg of a soil sample, comprising the following steps:

- 35
- preparation of the soil sample ;
 - optional dilution of the sample prepared ;
 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

19. Method of analysis according to Claim 18, for the analysis of amounts of less than or equal to 0.05 mg/kg.
- 5 20. Method of analysis according to either of Claims 18 and 19, for the analysis of amounts of less than or equal to 0.005 mg/kg.
21. Method according to any one of Claims 18 to 20, for which the sample is chosen from a clayey, sandy or chalky soil.
- 10 22. Method according to any one of Claims 18 to 21, for which the preparation is an extraction of the soil sample.
23. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.1 mg/m³ of an air sample, comprising the following steps:
- 15
 - preparation of the sample ;
 - optional dilution of the sample prepared ;
 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).
- 20 24. Method according to either of Claims 23 and 24, for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.01 mg/m³ of sample.
- 25 25. Method according to either of Claims 23 and 24, for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.001 mg/m³ of sample.
26. Method according to any one of Claims 23 to 25, for which the preparation is a trapping.
- 30 27. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.00005 mg/kg of a sample of a human body fluid, comprising the following steps.
- 35
 - preparation of the sample ;
 - optional dilution of the sample prepared ;
 - direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

28. Method according to either of Claims 27 and 28, for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.000005 mg/kg of sample.

5 29. Method according to either of Claims 27 and 28, for analyzing one or more pesticidal compounds present in amounts of less than or equal to 0.000005 mg/kg of sample.

30. Method according to any one of Claims 27 to 29, for which the sample is chosen from human blood and human urine.

31. Method for analyzing one or more pesticidal compounds present in amounts of less than or equal to 1 mg/kg of a sample of animal products or tissues, comprising the following steps:

- 15 - preparation of the sample of animal products or tissues ;
- optional dilution of the sample prepared ;
- direct analysis of the optionally diluted sample by high performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS).

20 32. Method of analysis according to Claim 31, for the analysis of amounts of less than or equal to 0.01 mg/kg.

33. Method of analysis according to either of Claims 31 and 32, for the analysis of amounts of less than or equal to 0.001 mg/kg.

25 34. Method according to any one of Claims 31 to 33, for which the sample is chosen from milk, eggs, liver, kidneys, fats and muscle.

35 35. Method according to any one of Claims 31 to 34, for which the preparation is an extraction of the animal products or tissues.

36. Method according to any one of Claims 13 to 17, for which the plant tissues are replaced with converted agrofood products.

37. Method according to any one of Claims 1 to 36, comprising a step consisting of dilution of the sample prepared.

38. Method according to any one of Claims 1 to 37, for the simultaneous analysis of several pesticidal compounds.

39. Method according to any one of Claims 1 to 38, for the analysis of one or more fungicidal, herbicidal, insecticidal or growth-regulating compounds.

5 40. Method according to any one of Claims 1 to 39, for the analysis of fungicidal compounds chosen from phosphorous acid or a derivative thereof ; phosphonic acid or a derivative thereof.

10 41. Method according to Claim 40, for the analysis of fosetyl or of one or more salts thereof.

42. Method according to Claim 41, for the analysis of fosetyl-Al.

15 43. Method according to any one of Claims 1 to 42, for the simultaneous analysis of phosphorous acid and of fosetyl-Al.

44. Method according to any one of Claims 1 to 43, for which the dilution is carried out in an aqueous solvent, which may be acidified, or in an organic solvent, which may be acidified, or in a mixture of such solvents.

20 45. Method according to Claim 44, for which the aqueous solvent comprises an acid chosen from formic acid, acetic acid or trifluoroacetic acid, or the organic solvent is chosen from acetonitrile or methanol.

MÉTODO PARA ANALIZAR EL ÁCIDO FOSFOROSO, EL FOSETIL-AL,
O AMBOS SIMULTÁNEAMENTE.

DESCRIPCIÓN

La presente invención se refiere al ámbito del análisis de compuestos plaguicidas.

5 Tales métodos son útiles para monitorizar el comportamiento de tales compuestos plaguicidas una vez que se han aplicado, y tales métodos son también útiles durante los procedimientos de autoridad de la mercadotecnia para tales compuestos plaguicidas.

Los compuestos que se pueden analizar por medio del método de análisis de acuerdo con la invención son compuestos que son útiles para la protección de las plantas y también
10 algunos de los compuestos que son metabolitos de esos compuestos biológicamente activos. Algunos de estos metabolitos también pueden mostrar actividades biológicas.

Se conocen métodos para analizar los compuestos plaguicidas. En particular, se conoce un método analítico para determinar los residuos de fosetil-Al y su principal metabolito, el ácido fosforoso, en el agua potable o en las aguas superficiales.

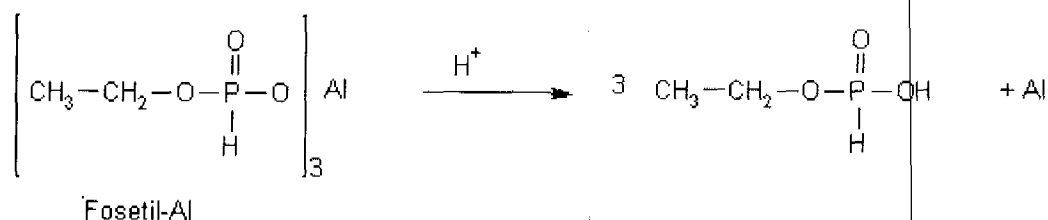
15 Tal método utiliza (trimetilsilil)diazometano (TMSD) como un agente para obtener derivados.

Generalmente, tal método comprende las etapas siguientes:

- Concentración de muestras de agua;
- Derivación con (trimetilsilil)diazometano de una alícuota de la muestra
20 concentrada (sustitución de un átomo de hidrógeno con un grupo metilo);
- Purificación de la muestra derivada mediante el reparto líquido-líquido con diclorometano.

El análisis se lleva a cabo mediante cromatografía de gases en una columna semicapilar por medio de un detector fotométrico de llama (FPD) en el modo fosforoso y la
25 cuantificación utiliza como referencia estándares externos. También es posible utilizar un detector termoiónico.

Este conocido método se lleva a cabo de acuerdo con el esquema 1 siguiente:



Con este método, los límites de detección (los LDD) son los siguientes:

- Para el fosetil-Al:
 - 0,05 µg/l para el agua potable (agua corriente o mineral);
 - 0,05 µg/l para las aguas superficiales (agua de río);
- Para el ácido fosforoso:
 - 0,7 µg/l para el agua potable (agua corriente o mineral);
 - 2,5 µg/l para las aguas superficiales (agua de río).

Con este método, los límites de cuantificación (los LDC) son los siguientes:

- Para el fosetil-Al:
 - 0,1 µg/l para el agua potable (agua corriente o mineral);
 - 1 µg/l para las aguas superficiales (agua de río);
- Para el ácido fosforoso:
 - 2,0 g/l para el agua potable (agua corriente o mineral);
 - 4,0 g/l para las aguas superficiales (agua de río).

Este conocido método de análisis se ha utilizado para los sustratos siguientes: agua mineral, agua corriente y agua del río Ródano. Se ha validado el método de análisis para diversos tipos de agua por medio del análisis de muestras de control sin tratar enriquecidas en fosetil-Al y en ácido fosforoso hasta los límites de cuantificación y hasta 10 veces estos límites.

El análisis de estas muestras enriquecidas ofrece unos valores de tasas de recuperación para el fosetil-Al o para el ácido fosforoso que son comparables a los valores teóricos esperados.

Como otro conocido método de análisis, se debe mencionar un método para analizar los residuos de fosetil-Al o de ácido fosforoso presentes en los suelos tomados en Chazay d'Azergues (Francia), en Goch (Alemania) y en Sevilla (España).

Este método por sí también utiliza (trimetil-silil)diazo-metano. También se lleva a cabo de acuerdo con el esquema 1.

En el transcurso de este método, los residuos de fosetil-Al y de ácido fosforoso se extraen de muestras de suelos mediante agitación en presencia de una disolución de tampón con amoníaco y, luego, los cationes que hay presentes se retiran de los extractos por medio de una resina de intercambio iónico y se evapora el agua de las muestras. Finalmente, los extractos obtenidos se derivan mediante la acción del (trimetilsilil)diazometano.

Posteriormente, se lleva a cabo la cuantificación mediante cromatografía de gases en una columna semicapilar con un detector fotométrico de llama (en el modo fosforoso) con un estándar externo.

El límite de cuantificación (LDC) de este método es de 0,100 mg/kg para cada uno de los compuestos.

Las muestras de control se enriquecieron en fosetil-Al o en ácido fosforoso hasta el límite de cuantificación y también por encima 100 veces este límite.

Otro método conocido para analizar residuos se refiere al análisis de los residuos de fosetil-Al y de ácido fosforoso en muestras vegetales, obtenidas de frutas y de vegetales.

Este método también utiliza el (trimetilsilil)diazometano. También se lleva a cabo de acuerdo con el esquema 1.

En el transcurso de este método, los residuos de fosetil-Al y de ácido fosforoso se extraen de las muestras vegetales moliéndolas en una mezcla de agua y acetonitrilo. Posteriormente, los extractos se purifican con un cartucho C18 y, luego, se obtienen derivados mediante la acción del (trimetilsilil)diazometano.

Posteriormente, se lleva a cabo la cuantificación mediante cromatografía de gases en una columna semicapilar con un detector fotométrico de llama (en el modo fosforoso) con un estándar externo.

El límite de cuantificación de este conocido método es de 0,50 mg/kg para cada uno de los productos, con la excepción del lúpulo, para el cual este límite es de 2,0 mg/kg para el fosetil-Al y de 20,0 mg/kg para el ácido fosforoso.

Este método se ha utilizado en muestras de racimos de uvas, de naranjas, de plátanos, de fresas, de lechuga y de pepinos. Las muestras de control se enriquecieron, en concreto, hasta el límite de cuantificación.

Otro método conocido para analizar residuos se refiere al análisis de los residuos de fosetil-Al o de ácido fosforoso en tejidos de animales o en productos de origen animal, como la leche, la carne vacuna, los riñones vacunos, el hígado vacuno o los huevos.

De acuerdo con este estudio, los residuos de los compuestos a analizar se extraen de las muestras mediante una doble molienda en una mezcla de agua/acetonitrilo (50/50, 20/80 para la leche).

Posteriormente, se purifica una alícuota del extracto por medio de un cartucho C18 (excepto para la leche). Posteriormente, del extracto purificado se obtienen derivados con una disolución de TMSD.

Este método de análisis también sigue el esquema 1.

La cuantificación se lleva a cabo mediante cromatografía de gases en una columna DB Wax con un detector fotométrico de llama en el modo fosforoso.

Los límites de cuantificación son los siguientes:

- 0,50 mg/kg para el fosetil-Al y el ácido fosforoso en la carne vacuna, el riñón vacuno, el hígado vacuno y los huevos;
- 0,10 mg/kg para el fosetil-Al y el ácido fosforoso en la leche.

Para este método, se prepararon y se analizaron muestras de control sin tratar, junto con muestras enriquecidas hasta el límite de cuantificación y también hasta varias veces por encima de este límite.

Los métodos conocidos de análisis que se acaban de mencionar cumplen las disposiciones de la directriz europea n.º 46 de 1996 (96/46/EC de 16 de julio de 1996), en particular con respecto a las características siguientes:

- Para cada uno de los sustratos y cada nivel:
 - La media de las tasas de recuperación debe estar entre el 70% y el 110%;
 - La repetibilidad, expresada como un coeficiente de variación (proporción de la desviación estándar a la media de la muestra referida, expresada como un porcentaje) debe ser, a lo sumo, el 20 %.
- Para cada uno de los sustratos, el coeficiente de variación total (incluidos todos los niveles) debe ser, a lo sumo, el 20 %.

Otro método para analizar el fosetil-Al se describe en un artículo titulado *Rapid determination of fosetyl-Al residues in lettuce by liquid chromatography/electrospray tandem mass spectrometry* (Hernández et al., *Journal of AOAC International*, Vol. 86, n.º. 4, 2003).

El método descrito se refiere a la cuantificación de los residuos de fosetil-Al en las muestras vegetales que se obtienen de las lechugas. El método requiere una etapa que consiste en la extracción con agua por medio de un mezclador de gran velocidad, seguida de la inyección de un extracto diluido 5 veces en una cromatografía de fase líquida.

Por lo tanto, el fosetil-Al se cuantifica mediante una cromatografía líquida acoplada a una espectrometría de masas en tandem por electropulverización después de añadir acetato de tetrabutilamonio como un agente de emparejamiento iónico.