Single- and Multiple-laboratory Validation of LC-MS/MS Method for Simultaneous Determination of Fosetyl-Al and Phosphonic Acid in Cereal Grains and Analysis of Rice, Wheat and Barley

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Abstract

Background and objective: In Japan, the residue definition for fosetyl-Al was “sum of fosetyl-Al and phosphonic acid expressed as fosetyl-Al” and its current provisional MRL in cereals was under review. For establishment and enforcement of fosetyl-Al MRL in cereals, a new analytical method for fosetyl-Al and phosphonic acid in cereals should be developed and validated.

Methods: The new method involved water extraction, clean-up using tandem cation- and anion-exchange mini-columns, and determination by LC-MS/MS. It was validated in single laboratory and multiple laboratories. Using the method, 41 samples of rice, wheat and barley were analyzed.

Results: In the multiple-laboratory validation: repeatability and reproducibility for three concentrations of fosetyl-Al and phosphonic acid were in ranges of 4.8–20% and 5.9–34%; calculated sum of fosetyl-Al and phosphonic acid, expressed as fosetyl-Al, showed good recoveries; linearity was observed for fosetyl-Al and phosphonic acid in ranges of 0.005–0.4 and 0.025–2.0 mg/kg; and specificity was sufficient. The method was verified for rice matrices. In 41 samples, phosphonic acid was detected up to 0.2 mg/kg while fosetyl was not.

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**Conclusion**: The method was successfully validated with high precision, accuracy, linearity, and specificity and capable of analyzing fosetyl-Al and phosphonic acid with the practical LOQ of 0.01 and 0.05 mg/kg. The LOQs and concentrations of phosphonic acid in samples indicate that a potential MRL would be 0.5 mg/kg for fosetyl-Al in cereals.

**Highlights**: The validated method was simpler than many methods and did not require derivatization or matrix matched or isotopically labeled internal standards.
Fosetyl and its aluminium salt (fosetyl-Al) are systemic fungicides registered in many countries for control of oomycete and ascomycete fungi and some plant pathogenic bacteria in fruit trees, vegetables and ornamental plants. Fosetyl-Al is registered as pesticide by the Ministry of Agriculture, Forestry and Fisheries in Japan (Ministry of Agriculture). The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) first evaluated fosetyl-Al in 2017 and noted that following the use of fosetyl-Al, it was “readily and rapidly hydrolyzed to phosphonic acid and ethanol in plants and soil.” The 2017 JMPR also indicated that phosphonic acid accounted for >80% of the residue, with far less fosetyl-Al present, in treated crops. Considering that phosphonic acid and fosetyl-Al are toxicologically similar, the 2017 JMPR established a residue definition for plant commodities for compliance with maximum residue limits (MRLs) and for dietary exposure assessment to be “sum of fosetyl, phosphonic acid and their salts, expressed as phosphonic acid” (1).

On the basis of similar results, the Ministry of Health, Labour and Welfare of Japan (Ministry of Health), responsible for setting MRLs for pesticides (2), established a residue definition for MRL compliance and for dietary exposure assessment to be “sum of fosetyl-Al and phosphonic acid expressed as fosetyl-Al” under the Food Sanitation Act (3). In other countries, the residue definitions for fosetyl-Al are: in the European Union, “sum of fosetyl, phosphonic acid and their salts expressed as fosetyl” (4); and in the USA (5) and Canada (6), “fosetyl-Al”. The Ministry of Health decided to revise the provisional MRL of 0.5 mg/kg for fosetyl-Al in cereal grains to 0.01 mg/kg because fosetyl-Al was not allowed for use on cereal grains in Japan. A number of countries questioned the Ministry of Health about this decision and requested either explanation or reconsideration. Consequently, the Ministry of Health suspended the revision and maintained the provisional MRL of 0.5 mg/kg.

Phosphonic acid, included in the residue definition in Japan, is not registered as pesticide but is registered as fertilizer (7) for use on plants in Japan. Therefore, phosphonic acid may arise in plant commodities from the fertilizer uses of phosphonic acid as well as from natural occurrence. On many occasions in Europe, phosphonic acid was reported to be frequently detected in organic produce (8, 9).
Many analytical methods were developed for quantitation of fosetyl-Al and phosphonic acid. Fosetyl-Al is known to dissociate in water to form O-ethyl phosphonate and aluminium cation, and then O-ethyl phosphonate is hydrolyzed to phosphonic acid and ethanol. The 2017 JMPR reviewed a number of analytical methods and reported that the LOQ of fosetyl-Al and phosphonic acid were, in general, in the range of 0.05–0.5 and 0.1–0.5 mg/kg, respectively, for gas chromatography with flame photometric detection (GC-FPD) and gas chromatography with nitrogen-phosphorus detection (GC-NPD) methods after methylation (1). An liquid chromatography with tandem mass spectrometry (LC-MS/MS) method patented by Bayer CropScience, a part of which was reviewed by the JMPR, achieved low LOQs: an LOQ of 0.01 mg/kg for fosetyl-Al and 0.1 mg/kg for phosphonic acid, using the matrix-matched standards (10). A multi-residue quick polar pesticides method (QuPPe method) used for enforcement of MRLs for fosetyl-Al and phosphonic acid in the European Union requires an isotopically labeled internal standard, and examples of LOQ include 0.02 mg/kg for fosetyl-Al in barley and 0.2 mg/kg for phosphonic acid in rice (11). Recently, newer methods were developed based on the QuPPe method and single-laboratory validated for some matrices, mostly fruits, with the LOQ of 0.01 mg/kg or lower, but they also use isotopically labeled internal standards (12, 13).

The official analytical methods of the Ministry of Health separate methylated analytes by GC coupled with various instruments, such as FPD, NPD, and MS (14). The LOQ is 0.5 mg/kg for both analytes: fosetyl-Al and phosphonic acid. A method used by the manufacturer in the supervised residue trials in Japan (15) employed methylation and analysis using GC-FPD with the LOQ in a range of 0.04–2.0 mg/kg for fosetyl-Al. However, residue trials were not conducted on cereal grains due to the lack of approval for use on cereal grains and therefore the applicability of this method to cereal grains was unknown. All of these analytical methods are not sufficiently sensitive or easy for enforcing the MRL of 0.01 mg/kg for the sum of fosetyl-Al and phosphonic acid, expressed as fosetyl-Al. In the European Union, temporary MRL for cereals is 2 mg/kg which, according to the European Union, "indicates lower limit of analytical determination" (4).

The Ministry of Agriculture serves also as an authorized importer of wheat, barley and rice grains to Japan, and it is important for this Ministry to maintain good...
relations with trading partners’ governments for smooth importation. Therefore, the Ministry of Agriculture decided to develop and validate an analytical method for fosetyl-Al and phosphonic acid in cereal grains to be useful for regulatory analysis, according to the OECD Guidance document on pesticide residue analytical methods (16) and Codex Guidelines on good laboratory practice in pesticide residue analysis (CXG 40-1993) (17), which describe method validation by conducting recovery tests at the proposed LOQ level and either ten times the proposed LOQ or MRL level. The new method should be much more sensitive than the methods of the Ministry of Health and easier and more commonly usable than other sensitive methods. The proposed LOQ was set to 0.01 mg/kg in cereal grains and the value of 0.01 mg/kg was common for the enforcement of MRLs for pesticide residues, except in the case for highly toxic pesticides. The Ministry of Agriculture further decided to analyze rice, wheat and barley, domestically produced without using those pesticides or fertilizers containing fosetyl-Al or phosphonic acid, or imported, to see the levels of phosphonic acid in these cereal grains. The method and analytical results will help the Ministry of Health to determine if it would be appropriate to establish an MRL for fosetyl-Al in cereal grains at the level of 0.01 mg/kg.

Methods

Sample Materials

Grains of wheat, six-row barley, and hull-less barley domestically grown in 2018 and wheat domestically grown in 2019 were used as blank materials for preparing spiked samples for validation of the new method. Additionally, brown rice from rice grown in 2019 was used for verification of the applicability of the method to rice matrices. It was confirmed that the wheat, six-row barley and hull-less barley were grown conventionally without using any pesticides or fertilizers containing fosetyl-Al or phosphonic acid. The rice (var. Nikomaru) was certified organic produce without using any chemical pesticides or chemical fertilizers.

Six grain samples of the above were analyzed for fosetyl and phosphonic acid. Additionally, 35 samples of wheat and rice were used for the determination of fosetyl and phosphonic acid: 10 samples of wheat grains imported from the USA; 15 samples of white rice imported from USA or Thailand; and 10 samples of brown rice domestically produced.
Apparatus

(a) **LC-MS/MS.**—HPLC Exion LC [SCIEX]; equipped with MS 5500 Q TRAP (SCIEX); and LC column L-column 2 ODS Metal-free, 2.0 mm i.d. × 150 mm, particle size 5 μm (Chemicals Evaluation and Research Institute Japan).

Other LC-MS/MS instruments used in the multiple-laboratory validation:
(b) **HPLC.**—Shimadzu Nexera series; Waters Acquity series; or Agilent 1260 Infinity series.
(c) **MS.**—AB Sciex QTRAP 5500; AB Sciex 5500+; Waters Xevo TQ-S micro; or Waters Xevo TQ-XS; Shimadzu LCMS 8060; or Agilent-6495C
(d) **Ultra-centrifugal mill.**—Retsch ZM 200 (Verder-Scientific).
(e) **Vacuum manifold system.**—GL Sciences Inc.
(f) **Aspirator.**—GL Sciences Inc.
(g) **Shaker.**—Elvis Shaker (Sugiyama-Gen).
(h) **Centrifuge.**—H 700 F (Kokusan).
(i) **Evaporator.**—Rotary evaporator (Eyela).
(j) **Ultrasonic bath.**—Ultrasonic Cleaner VS-D100 (Velvo-Clear).
(k) **Cation-exchange mini-column.**—Oasis MCX 500 mg (Waters).
(l) **Anion-exchange mini-column.**—InertSep MA-1 500 mg (GL Sciences).
(m) **Cartridge adapter for mini-column.**—GL Sciences Inc.
(n) **Reservoir for mini-column.**—GL Sciences Inc.

Reagents

(a) **Fosetyl-Al.**—Purity 99.4% (Fujifilm Wako Pure Chemical).
(b) **Phosphonic acid.**—Purity 98.0% (Dr. Ehrenstorfer).
(c) **Ion-pair reagent for LC/MS.**—0.5 mol/L dibutylammonium acetate in water (hereafter referred to as 0.5 mol/L DBAA) (Tokyo Chemical Industry).
(d) **Water.**—Ultra-pure water (Milli-Q water).
(e) **Methanol.**—HPLC grade (Kanto Chemical Co., Inc.)
(f) **36.0% Hydrochloric acid (35.0–37.0%, w/w).**—Japan industrial standard (JIS) special grade (Koso Chemical Co. Ltd).
(g) **Diatomaceous earth.**—Celite 545 (Kanto Chemical Co., Inc.)

**Preparation of Solutions**
(a) 0.01 mol/L hydrochloric acid in methanol.—Measure 500 mL methanol using a 500 mL graduated cylinder, and pour it into a 500 mL Erlenmeyer flask, to which add 0.5 mL 36% hydrochloric acid using a 0.5 mL volumetric pipette.

(b) 10 mmol/L DBAA solution.—Measure 980 mL water using a 1000 mL graduated cylinder, and pour it into a 1000 mL Erlenmeyer flask, to which add 20 mL 0.5 mol/L DBAA using a 20 mL volumetric pipette.

(c) Fosetyl-Al stock solution (500 μg-fosetyl-Al/mL).—Weigh accurately 25 mg fosetyl-Al in a glass weighing boat, add water using a Komagome pipette to dissolve it, transfer the solution to a 50 mL volumetric flask, and dilute to 50 mL with water.

(d) Fosetyl-Al standard solution A (0.4 μg-fosetyl-Al/mL).—Pipette 1 mL fosetyl stock solution into a 25 mL volumetric flask and dilute exactly to 25 mL with water to prepare 20 μg/mL solution. Pipette 1 mL 20 μg/mL solution into a 50 mL volumetric flask and dilute to 50 mL with water.

(e) Phosphonic acid stock solution (500 μg-phosphonic acid/mL).—Weigh accurately 25 mg phosphonic acid in a glass weighing boat, add water using a Komagome pipette to dissolve it, transfer the solution to a 50 mL volumetric flask, and dilute to 50 mL with water.

(f) Phosphonic acid standard solution A (2.0 μg-phosphonic acid/mL).—Pipette 1 mL phosphonic acid stock solution into a 25 mL volumetric flask and dilute exactly to 25 mL with water to prepare 20 μg/mL solution. Pipette 5 mL 20 μg/mL solution into a 50 mL volumetric flask and dilute to 50 mL with water.

(g) Standard solution B (0.05 μg-fosetyl-Al + 0.25μg-phosphonic acid/mL).—Pipette 2.5 mL fosetyl-Al standard solution A and 2.5 ml phosphonic acid standard solution A into a 20 mL volumetric flask and dilute to 20 mL with 10 mmol/L DBAA solution.

(h) Calibration solutions.—Prepare solutions for the calibration curve covering a range of 0.000125 μg/mL to 0.01 μg/mL fosetyl-Al, and 0.000625 μg/mL to 0.05 μg/mL phosphonic acid. Prepare the calibration solutions anew daily by series of dilution of standard solution B with 10 mmol/L DBAA solution.

**Analytical Method**

The new method using LC-MS/MS was developed by the Japan Food Research Laboratories (JFRL), accredited according to ISO/IEC 17025-2005 for pesticide residue analysis.
Comminute all cereal grain samples using an ultra-centrifugal mill with a 1.00 mm screen and put each comminuted grain sample into a thick polyethylene bag.

Weigh 10.0 g comminuted grain sample into a 250 mL screw-cap polypropylene centrifuge tube. Add 100 mL water to the centrifuge tube and mix the content using a shaker for 60 min. Centrifuge at 1650 × g for 5 min and pour the supernatant on a Kiriyama funnel in which glass fiber filter paper and 2 cm Celite 545 layer were placed. Using an aspirator, filter the liquid and receive the filtrate in a 200 mL volumetric flask. Rinse the centrifuge tube with 50 mL of water, pour it on the same Kiriyama funnel, and receive the filtrate in the same volumetric flask. Dilute the content of the volumetric flask exactly to 200 mL with water.

Load 1 mL of the extract in the cation-exchange mini-column (top part of the connected ion-exchange mini-columns). Open the stopcock and drain liquid until the solid surface starts to show. Then pour approximately 5 mL water in the cation-exchange mini-column and drain liquid in the same manner two times. Close the stopcock and remove the cation-exchange mini-column and cartridge adapter from the vacuum manifold system. Pour 5 mL methanol into the anion-exchange mini-column, open the stopcock and drain liquid until the solid surface starts to show, and then close the stopcock. Connect a reservoir to the top of the anion-exchange mini-column and place a ground glass joint 50 mL centrifuge tube under the mini-column. Pour 15 mL 0.01 mol/L hydrochloric acid in methanol on top of the anion-exchange mini-column, open the stopcock and elute until the solid surface starts to show. Remove the ground glass joint 50 mL centrifuge tube and place it in a rotary evaporator. Evaporate the elute to approximately 1 mL below 40°C and then dry the concentrate under nitrogen flow at the room temperature. Add 2 mL 10 mmol/L DBAA solution into that 50 mL centrifuge tube using a 2 mL volumetric pipette and place the tube in an ultrasonic bath. Pipette approximately 1 mL from liquid in that 50 mL centrifuge tube using a Pasteur pipette, filter it through a syringe-filter, and transfer it to a vial with screw cap for LC-MS/MS as a test solution.

Inject 5 µL of the cleaned-up sample solution onto a metal-free ODS column of HPLC. Set the condition of the HPLC as follows:

Column temperature.—30°C.

Flow rate to: 0.2 mL/min.

Elute the analytes with a mixture of 10 mmol/L DBAA solution and methanol (9:1, v/v) for 20 min. Determine fosetyl and phosphonic acid by tandem mass
spectrometry at the following conditions:

Ionization method.—Electrospray ionization (ESI negative ion mode).

Ionization voltage.— –4500 V.

Ionization temperature.—700°C:

Nebulizer gas.—Nitrogen gas at 0.55 MPa.

Precursor ion (m/z) and product ion (m/z).—109 and 63 for fosetyl; and 81 and 79 for phosphonic acid.

Inject 5 μL each of prepared calibration solutions into LC-MS/MS in sequence, record each chromatogram and peak areas of fosetyl and phosphonic acid. Plot the peak area of calibration solution against the concentration to draw calibration curves for fosetyl-Al and phosphonic acid. Despite the analytical method determines fosetyl rather than fosetyl-Al, calculate the concentration of fosetyl-Al as it is the compound included in the residue definition in Japan, using the calibration curve of fosetyl-Al.

For cereal grain samples, calculate the concentrations of fosetyl-Al and those of phosphonic acid from the respective concentrations in the test solutions as follows:

\[ C \text{ (mg/kg)} = C_1 \times \frac{2 \text{ mL}}{1 \text{ mL}} \times \frac{200 \text{ mL}}{10.0 \text{ g}} \]

where, \( C \) is the concentration of fosetyl-Al or phosphonic acid in the cereal grain sample; and \( C_1 \) is the concentration of fosetyl-Al or phosphonic acid in test solution calculated using the respective calibration curves.

Preparation of Spiked Samples

Add to each comminuted blank material, amounts of the fosetyl-Al and phosphonic acid standard solutions to the predetermined concentrations, as shown in Table 1: at the proposed LOQ (low level), at ten times the proposed LOQ (high level), and at the level in-between (three or four times, respectively for fosetyl-Al or phosphonic acid; middle level). Shake each bag thoroughly and press it by hands to crush lumps until no lumps were observable, after which check the homogeneity.

Single-Laboratory Validation (SLV)

The method was validated by the JFRL, developer of the method, for wheat and barley according to the OECD guidance document (16) and Codex guidelines (17), taking into consideration AOAC INTERNATIONAL Guidelines for Single-Laboratory Validation (18), and Codex Alimentarius Commission Procedural manual, 27th Edition (19).

After the homogeneity of the analytes in each sample simultaneously spiked with fosetyl-Al and phosphonic acid at the low level (see Table 1) was ensured, analyze
spiked materials. For estimating intermediate relative standard deviation (RSD), analyze each sample in duplicates on each of 5 consecutive working days, totaling 10 analyses (2/day × 5 days).

**Multiple-Laboratory Validation**

For pesticide residue analytical methods for enforcement purposes, in addition to in-house validation in the laboratory where the method was developed, independent laboratory validation (ILV) was necessary. As the method was intended for use in enforcement purposes, ILV was conducted in multiple laboratories. In order to ensure the objective nature of the validation, spiked samples were prepared at the JFRL and sent to the participating laboratories after checking the homogeneity. The study design followed as much as possible the AOAC International Guidelines for Collaborative Study (20). A total of nine laboratories from three countries participated in this validation (seven from Japan, one from Thailand and one from the USA).

After confirming the homogeneity of the analytes in each sample, send the following samples to the nine participating laboratories together with the clean-up mini-columns, standard operating procedure (SOP) and reporting template. Store the received samples in a refrigerator (2 to 6°C) until analysis in each laboratory.

(a) **Eight test samples.**—Duplicate samples × 3 concentration levels and two blank samples (each approx. 20 g, labeled with 3-digit random numbers).

(b) **One blank sample.**—For procedural recovery tests (approx. 100 g).

**Verification of the Applicability to Rice**

Verification of the method for analysis of rice samples was conducted in the JFRL.

Spike the comminuted blank brown rice with fosetyl-Al and phosphonic acid at the low and high levels as shown in Table 1. After confirming the homogeneity, analyze the samples (2/day × 5 days).

**Results and Discussions**

**SLV**

The analytical method was validated for three matrices: wheat, six-row barley, and hull-less barley.

Calibration curves were prepared for fosetyl-Al and phosphonic acid in the ranges of 0.000125 to 0.0025 μg/mL and 0.000625 to 0.0125 μg/mL, respectively. These ranges are equivalent to 0.005–0.1 mg/kg of fosetyl-Al and 0.025–0.5 mg/kg of
phosphonic acid in comminuted samples. The linearity was demonstrated with $r^2$ of ≥0.995 in these ranges.

The results of recovery tests of wheat, six-row barley, and hull-less barley spiked at 0.01 mg/kg fosetyl-Al and 0.05 mg/kg phosphonic acid and analyzed in five consecutive working days are shown in Table 2. The mean recoveries, as calculated as percentage of the spiked concentrations, were 84–94% and 88–96% for fosetyl-Al and phosphonic acid, respectively in these matrices. The repeatability RSD ($RSD_r$) values were 3.9–7.7% and intermediate RSD ($RSD_i$) values were 6.3–10.5%. The values meet the requirements for pesticide residue analysis in the Codex Guidelines (17), which specify that in the concentration range of >0.01 and ≤0.1 mg/kg, or of >0.001 and ≤0.01 mg/kg, the recovery range should be 70–120% or 60–120%, respectively. The 27th Edition of the Codex Alimentarius Commission Procedural Manual (19) specifies that at the level of 0.1 mg/kg or 0.01 mg/kg, the recovery range should be 80–110% or 60–115%, respectively.

The LOQs for fosetyl-Al calculated as ten times the standard deviation (SD) of analytical results ($n = 7$) of wheat and barley freshly spiked at the low level on the day of analysis were equivalent to 0.0074 mg/kg in wheat and 0.0054 mg/kg in barley, lower than the proposed LOQ of 0.01 mg/kg. For phosphonic acid, the calculated LOQs in the same manner as for fosetyl-Al were equivalent to 0.044 mg/kg in wheat and 0.042 mg/kg in barley. Due to the low S/N ratios observed for the phosphonic acid calibration solutions, it was not possible to lower the LOQ of phosphonic acid close to 0.01 mg/kg and therefore a new proposed LOQ for phosphonic acid was set to 0.05 mg/kg in cereal grains.

It was concluded that this method was applicable at concentrations, ≥0.01 mg/kg of fosetyl-Al and ≥0.05 mg/kg of phosphonic acid in cereal grains, i.e., the proposed LOQ of 0.01 mg/kg for fosetyl-Al was achievable. The ratio of the LOQ levels of the two analytes coincided closely with their ratios in the residues in plant metabolism studies evaluated by the 2017 JMPR (<20% as fosetyl and ≥80% as phosphonic acid) (1).

This method was simpler and more commonly usable with less matrix effects compared to other recent methods as it employed water extraction, smaller number of reagents, and readily available clean-up mini-columns, without derivatization, matrix matched standards or isotopically labeled internal standards.

MLV
Before sending the spiked samples to the participating laboratories, the homogeneity of the wheat samples spiked at the three different levels was tested at the JFRL by analyzing fosetyl-Al and phosphonic acid in ten portions randomly taken from each spiked sample because for preparing spiked samples for distribution to the laboratories required a larger quantity of grain samples. The LOQs calculated as ten times the standard deviation (SD) of analytical results (n = 7) of wheat freshly spiked at the low level on the day of analysis were equivalent to 0.0041 mg/kg for fosetyl-Al and 0.044 mg/kg for phosphonic acid. The concurrent procedural recoveries of freshly spiked fosetyl-Al and phosphonic acid at the low level in wheat were 83 and 90%, respectively.

The analytical results of the spiked wheat at three levels and kept at 5°C (low and medium levels, analyzed 2 days after; and high level, 6 days after spiking) are shown in Table 3. The RSD, values of these samples were in the range of 2.2–8.5%, indicating that these spiked samples were sufficiently homogeneous. Table 3 also shows that the recoveries of fosetyl-Al were 61–65% at the three spike levels. At the spike level of 0.01 mg/kg of fosetyl-Al, the recovery of 61% was barely within the acceptable range specified in the Codex requirements (≥60%) (17), but, at the spike level of 0.03 and 0.1 mg/kg, the recovery of 65% was lower than the lower end of the acceptable range in the Codex requirements (≥70%) (17). The recoveries of phosphonic acid were 89–91%, within the Codex acceptable range (70-110%) (17), confirming the relative stability of phosphonic acid as reported by the 2017 JMPR (1).

Assuming that the ratio of fosetyl of <20% and phosphonic acid of ≥80% in the residues in plant metabolism studies to be applicable to cereal grains, the calculated sum of fosetyl-Al and phosphonic acid, expressed in fosetyl-Al in the samples, in accordance with the residue definition in Japan, using the analytical results in Table 3, showed recoveries of 86–88%, within the Codex acceptable range.

As 2017 JMPR reported degradation of fosetyl-Al to phosphonic acid in the storage stability studies, and the mean recovery of fosetyl-Al from the wheat spiked at 0.01 mg/kg and stored was lower than the procedural recoveries (mean procedural recovery of 80% at the 0.01 mg/kg in MLV), we investigated if degradation of fosetyl-Al to phosphonic acid occurred during the processes after spiking, such as storage and early steps of analytical procedures. Only fosetyl-Al was added to blank wheat samples at a concentration of 0.5 mg/kg and the spiked samples were analyzed after storage for 3 days at 5°C. Table 4 indicates that after the storage for 3 days at 5°C, the mean
concentration of fosetyl-Al was 0.37 mg/kg in the samples, corresponding to 75% of the spiked level of 0.5 mg/kg. The mean concentration of newly formed phosphonic acid after the storage calculated by subtracting the mean concentration in the blank sample reached the level equivalent to 0.079 mg/kg of fosetyl-Al (16% of 0.5 mg/kg). After the storage for 3 days, 83% (75/91x100) of the spiked fosetyl-Al remained unchanged and 17% equivalent of the fosetyl-Al was estimated to be hydrolyzed to phosphonic acid.

The wheat samples simultaneously spiked with fosetyl-Al and phosphonic acid at the three spiking levels and analyzed after 2 (low and middle level) or 6 days (high level) after spiking were further stored at 5°C. They were analyzed at intervals up to 120 days. Concurrent procedural recoveries during the whole storage period at the low level were 65–87% for fosetyl-Al and 84–90% for phosphonic acid. Table 5 shows the mean analytical results of fosetyl-Al and phosphonic acid in duplicate samples (except at the initial timing, 10 samples) and “percent remaining” after certain intervals up to 120 days. Percent remaining values were calculated taking the mean analytical results at the initial timing (2 or 6 days after spiking) as 100% for determining stability of fosetyl-Al and phosphonic acid during storage at 5°C. During the storage from 2 or 6 days up to 120 days, 81–113% and 85–108% of the initial concentrations of fosetyl-Al and phosphonic acid, respectively, remained. These results indicated that, after shipment of the spiked samples, fosetyl-Al and phosphonic acid were stable during refrigeration at 5°C for at least 120 days with the sum of fosetyl and phosphonic acid also remaining unchanged. Fosetyl-Al degraded to less than 70% of the spiked level between the spiking and early period of storage of the spiked samples, as shown in Table 3.

Analytical results of the test samples were submitted from all nine participating laboratories. These laboratories did not use the identical analytical instruments. However, they demonstrated comparable linearity and range of the calibration and specificity.

Among nine participating laboratories, the results from two laboratories were not included in the statistical analysis because of their: (i) non-compliance with the SOP; and/or; (ii) unacceptably low recoveries.

The valid results were obtained from the other 7 laboratories. While the AOAC INTERNATIONAL Guidelines for Collaborative Study (20) require 8 laboratories, the guidelines also state that when using expensive instruments, the maximum of 5
laboratories are needed. Therefore, data obtained by 7 laboratories were statistically treated following the way described in the AOAC INTERNATIONAL Guidelines for Collaborative Study (20). For the analysis of these samples, the participating laboratories conducted procedural recovery tests with blank samples spiked on the day of analysis. The mean procedural recoveries in these seven laboratories were in a range of 65–110% (mean, 80%) and 77–104% (mean, 89%) at the low level spike; and 73–94% (mean, 79%) and 84–106% (mean, 90%) at the high level spike, for fosetyl-Al and phosphonic acid, respectively, all within the acceptable range of the Codex requirements (17). The recoveries for the sum of fosetyl-Al and phosphonic acid, expressed in fosetyl-Al, as per the residue definition in Japan, were in a range of 75–103% (mean, 88%) at the low level and 83–104% (mean, 89%) at the high level.

Among the analytical results of duplicate samples at three spike levels provided by the 7 laboratories, Cochran and Grubbs tests indicated that: for each of the 3 levels of phosphonic acid, one laboratory produced outliers; and for fosetyl-Al, another laboratory produced outliers for samples spiked at the medium level.

Table 6 shows the number of analytical results used for statistical analysis, either 12 or 14, for the three spike levels and the performance characteristics of the method calculated using these analytical results. Repeatability (RSD_r) and reproducibility (RSD_R) of fosetyl-Al at the low were 20 and 34%, respectively and they were within the acceptable levels of the Codex requirements (≤30 and ≤45%, respectively, between 0.001 and 0.01 mg/kg) (17). RSD_r of fosetyl-Al at the medium and high levels and phosphonic acid at all spiking levels were 4.8–20% and 6.5–11% respectively, within the Codex acceptable range (≤20% between 0.01 and 0.1 mg/kg and ≤15% between 0.1 and 1 mg/kg) (17). RSD_R of fosetyl-Al at the medium and high levels and phosphonic acid at all spiking levels were 23–24% and 5.9–13% within the Codex acceptable range (≤32% between 0.01 and 0.1 mg/kg and ≤23% between 0.1 and 1 mg/kg) (17). HorRat values were in a range of 0.29–1.54, meeting the AOAC guideline requirements of ≤2.0 (20). These performance characteristics meet the requirements for the determination of fosetyl-Al at or higher than 0.006 mg/kg and phosphonic acid at or higher than 0.05 mg/kg.

The calibration curves for fosetyl-Al and phosphonic acid showed high linearity ($r^2 \geq 0.995$) in ranges equivalent to 0.005–0.4 and 0.025–2.0 mg/kg in wheat grain, respectively. There were no interfering peaks observed at the retention time of each analyte when matrix blank samples or fortified samples were injected into HPLC.
Verification for Rice Analysis

Homogeneity of the two analytes in each of the spiked samples was confirmed with the RSD of 3.6–6.8%. The mean concurrent procedural recoveries of freshly spiked fosetyl-Al and phosphonic acid were 82 and 84%, respectively at the low level and 87 and 85%, respectively at the high level.

Table 7 shows the results of duplicate analysis of the two spiked samples for five consecutive working days at the JFRA. The recoveries were 83–88% for both fosetyl-Al and phosphonic acid; RSDr values 5.3–8.9%; and RSDi values 5.3–9.7%. The method was found to be also applicable to determine fosetyl-Al above 0.01 mg/kg and phosphonic acid above 0.05 mg/kg in brown rice.

Analytical results on Wheat, Barley, and Rice Grains

Using the method, domestically produced rice, wheat, six-row barley and hull-less barley, grown without using fosetyl or phosphonic acid were analyzed. No fosetyl was expected to be detected from these samples.

Figure 1 shows the typical multiple reaction monitoring (MRM) chromatograms of a standard solution containing both fosetyl-Al (0.01 mg/kg) and phosphonic acid (0.05 mg/kg), and brown rice sample, with the monitoring ions of m/z 109→63 and m/z 81→79. On the chromatogram of brown rice sample monitored with m/z 109→63, no fosetyl peak was observed. However, on the chromatogram of the same brown rice sample monitored with m/z 81→79, despite neither fosetyl-Al nor phosphonic acid was used during the cultivation, the peak of phosphonic acid was clearly observed. This peak was also observed in other brown and white rice samples as well as in barley and wheat samples.

In addition to the six samples of grains mentioned above, 35 samples of rice and wheat were also analyzed for fosetyl and phosphonic acid. Table 8 shows the analytical results of a total of 41 samples of cereal grains. In any of the analyzed samples, domestically produced or imported, no peak of fosetyl-Al was observed. However, the peak of phosphonic acid was observed in all the samples. Table 8 shows the values in parentheses where the peak was observed on the chromatograms but at levels below the proposed LOQ. In all the domestically produced cereal samples, either known to have been grown without using fosetyl-Al or phosphonic acid, or without such information, the phosphonic acid peak was observed in the chromatograms despite the concentrations were all below the proposed LOQ of 0.05 mg/kg. The results
confirmed that rice in Japan was produced in compliance with the regulation of fosetyl-Al, i.e., without using it on rice.

In all imported rice and wheat samples, the phosphonic acid peak was observed: up to 0.18 mg/kg in imported wheat; and up to 0.20 mg/kg in imported white rice. These concentrations correspond to up to 0.29 mg/kg expressed in fosetyl-Al, significantly higher than the planned revised MRL for fosetyl-Al at 0.01 mg/kg. There were no records concerning the use of fosetyl, fosetyl-Al or phosphonic acid during the cultivation of these imported cereal grains. Fosetyl-Al has not been registered for use on cereal grains in these exporting countries. However, phosphonic acid concentrations were generally higher in the imported cereal grain samples than those in domestically produced cereal grains.

Conclusions

The new method showed high precision, accuracy, linearity, and specificity and was found to be capable of analyzing fosetyl-Al and phosphonic acid with the practical LOQ of 0.01 mg/kg and 0.05 mg/kg, respectively, which implied that an MRL of 0.01 mg/kg for the sum of fosetyl-Al and phosphonic acid, expressed in fosetyl-Al, would be too low to enforce.

It was confirmed that phosphonic acid was present in domestically produced cereal grains (wheat, barley and rice) at or higher than 0.01 mg/kg, reaching 0.02 mg/kg, even when they were cultivated without using fosetyl-Al or phosphonic acid, and no fosetyl peak was observed in the chromatogram of any sample. The highest detected level of phosphonic acid in cereal grains imported from the countries where fosetyl-Al was not allowed for use on cereal grains was close to 0.3 mg/kg (0.287 mg/kg) in fosetyl-Al equivalents as defined in the residue definition in Japan. This also confirmed that an MRL for fosetyl-Al and phosphonic acid, expressed in fosetyl-Al, in cereal grains should be significantly higher than 0.01 mg/kg. Based on the data on 41 samples and taking into consideration the LOQ achievable for phosphonic acid, a potential MRL value would be 0.5 mg/kg, the same as the provisional MRL in Japan, or higher, even without legally permitted use of fosetyl-Al on cereal grains.

Acknowledgments

This research was funded by the Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan. The authors wish to thank the following collaborators, participants, and their associates for their cooperation in the validation:
Ayumu Nakamura, Japan Food Research Laboratories
Kazuhiro Ogura, Japan Food Research Laboratories
Eiko Nagasawa, Chemicals Evaluation Research Institute, Japan
Toshiyoshi Nakata, Hiyoshi Co., Ltd., Japan
Masayuki Kaneko, Japan Inspection Association of Food and Food Industry
Environment
Kazuhiro Kashiwabara, Incorporated Foundation Tokyo Kenbikyo-in
Eri Inagaki, Saika Technological Institute Foundation, Japan
Akihiro Iwaya, Overseas Merchandise Inspection Co., Ltd.
Yasuhami Suzuki, Overseas Merchandise Inspection Co., Ltd.
Hitoshi Tsuchiya, Japan Food Research Laboratories
Tomomi Ogawa, Japan Grain Inspection Association

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Figure Caption
Figure 1. MRM chromatograms of fosetyl and phosphonic acid in the standard solution and brown rice sample.
Table 1. Spiking levels used in the validation\textsuperscript{a}

| Spike level | Concn, mg/kg Fosetyl-Al | Phosphonic acid |
|-------------|-------------------------|----------------|
| Low         | 0.01                    | 0.05           |
| Medium      | 0.03                    | 0.2            |
| High        | 0.1                     | 0.5            |

\textsuperscript{a}The “low level” corresponded to the proposed LOQ values for fosetyl-Al and phosphonic acid, and the “high level” corresponded to ten times the proposed LOQ values.
Table 2. Single-laboratory validation of the method by recovery tests at the spiking levels of 0.01 mg/kg fosetyl-Al and 0.05 mg/kg phosphonic acid in wheat, six-row barley and hull-less barley grains\(^a\)

| Statistical values in the single-laboratory validation | Wheat (var. Nourin No.61) | Six-row barley (var. Shunrai) | Hull-less barley (var. Ichibanboshi) |
|--------------------------------------------------------|----------------------------|-------------------------------|-------------------------------------|
|                                                        | Fosetyl-Al                 | Phosphonic acid               | Fosetyl-Al                          | Phosphonic acid                 | Fosetyl-Al                          | Phosphonic acid                 |
| Mean recovery, %                                        | 94.4                       | 95.5                          | 88.2                                | 93.6                          | 83.5                                | 88.4                          |
| Repeatability (RSD\(_r\), %)                           | 6.8                        | 3.9                           | 7.1                                 | 5.7                           | 5.0                                 | 7.7                           |
| Intermediate reproducibility (RSD\(_i\), %)\(^b\)       | 10.5                       | 9.2                           | 7.5                                 | 8.3                           | 6.3                                 | 8.2                           |

\(^a\)The percent recoveries were calculated after subtracting the concentrations in the blank samples (fosetyl-Al, 0.0000 mg/kg in all samples; and phosphonic acid, 0.0097 mg/kg in wheat, 0.0193 mg/kg in six-row barley, and 0.0194 mg/kg in hull-less barley) from the respective analytical results.

\(^b\)Analyzed in duplicates on each of five consecutive working days (2/day × 5 days).
Table 3. Results of homogeneity test of fosetyl-Al, phosphonic acid, and the sum of fosetyl-Al and phosphonic acid, expressed as fosetyl-Al, in test samples of comminuted wheat grain (var. Shiroganekomugi) at three different spiking levels (10 samples each)a

| Spike, mg/kg | Fosetyl-Al | Phosphonic acid | Totalb | Fosetyl-Al | Phosphonic acid | Total | Fosetyl-Al | Phosphonic acid | Total |
|-------------|------------|----------------|--------|------------|----------------|-------|------------|----------------|-------|
| 0.01        | 0.006      | 0.053          | 0.082  | 0.019      | 0.187          | 0.288 | 0.065      | 0.462          | 0.730 |
| 0.05        | 0.004      | 0.0045         | 0.0067 | 0.0009     | 0.0041         | 0.0063 | 0.0041     | 0.024          | 0.038 |
| 0.08        | 0.065      | 0.0009         | 0.082  | 0.019      | 0.187          | 0.288 | 0.065      | 0.462          | 0.730 |
| 0.03        | 0.0041     | 0.0041         | 0.0063 | 0.0041     | 0.024          | 0.038 | 0.0041     | 0.024          | 0.038 |
| 0.2         | 0.065      | 0.0009         | 0.0063 | 0.0041     | 0.024          | 0.038 | 0.0041     | 0.024          | 0.038 |
| 0.32        | 0.187      | 0.0041         | 0.288  | 0.187      | 0.041          | 0.228 | 0.187      | 0.041          | 0.228 |
| 0.1         | 0.0065     | 0.0041         | 0.082  | 0.019      | 0.024          | 0.043 | 0.019      | 0.024          | 0.043 |
| 0.5         | 0.065      | 0.0041         | 0.082  | 0.019      | 0.024          | 0.043 | 0.019      | 0.024          | 0.043 |
| 0.82        | 0.288      | 0.0063         | 0.32   | 0.288      | 0.038          | 0.326 | 0.288      | 0.038          | 0.326 |

a The samples spiked at the low and medium levels were analyzed 2 days after spiking and those spiked at the high level were analyzed 6 days after spiking.

b “Total” refers to the sum of fosetyl-Al and phosphonic acid expressed as fosetyl-Al and was calculated by summing the concentration of fosetyl-Al and 1.44 times the concentration of phosphonic acid.

c Unadjusted for recovery.

d Percent recoveries were calculated after subtracting the blank value (fosetyl-Al: 0.0000 mg/kg and phosphonic acid 0.0079 mg/kg) from the respective analytical results.
Table 4. Stability of fosetyl-Al spiked in wheat samples and analyzed after 3 days of storage at 5°C (var. Shiroganekomugi)

| Spike, mg/kg | Fosetyl-Al | Phosphonic acid | Phosphonic acid in fosetyl-Al equiv.\(^a\) | Total\(^b\) |
|--------------|------------|----------------|------------------------------------------|-----------|
| Mean concn of triplicate analysis, mg/kg | 0.374 | 0.055\(^c\) | 0.079 | 0.453 |
| Mean recovery, % | 74.9 | — | 15.8\(^d\) | 90.7 |
| Ratio of fosetyl-Al in the total recovered, % | | | 82.6 (74.9/90.7×100) | |

\(^a\) Calculated by multiplying the concentration of phosphonic acid by 1.44.

\(^b\) “Total” refers to the sum of fosetyl-Al and phosphonic acid expressed as fosetyl-Al and was calculated by summing the concentration of fosetyl-Al and 1.44 times the concentration of phosphonic acid.

\(^c\) The concentration was calculated by subtracting the blank value, 0.0079 mg/kg, from the analytical results.

\(^d\) Calculated by dividing the mean concentration of phosphonic acid in fosetyl-Al equivalents by the spiking level of fosetyl-Al.
Table 5. Stability of fosetyl-Al and phosphonic acid in wheat samples stored at 5°C up to 120 days (average of the analytical results of two samples)\(^a\)

| Storage, days | Fosetyl-Al |  | Phosphonic acid |  | Total\(^b\) |  |
|---------------|-----------|---|----------------|---|-------------|---|
|               | Conc, mg/kg\(^c\) | % Remaining | Conc, mg/kg | % Remaining | Conc, mg/kg | % Remaining |
| Low level spike | 2\(^d\) | (0.0061) | (100) | 0.0527 | 100 | 0.0820 | 100 |
|                | 15 | (0.0055) | (90) | 0.0531 | 101 | 0.0820 | 100 |
|                | 20 | (0.0069) | (113) | 0.0522 | 99 | 0.0821 | 100 |
|                | 30 | (0.0060) | (98) | 0.0533 | 101 | 0.0828 | 101 |
|                | 58 | (0.0050) | (82) | (0.0479) | (91) | 0.0740 | 90 |
|                | 90 | (0.0055) | (90) | (0.0480) | (91) | 0.0746 | 91 |
|                | 120 | (0.0059) | (97) | 0.0559 | 106 | 0.0864 | 105 |
| Medium level spike | 2\(^d\) | 0.0194 | 100 | 0.187 | 100 | 0.288 | 100 |
|                | 15 | 0.0176 | 91 | 0.172 | 92 | 0.274 | 95 |
|                | 20 | 0.0216 | 111 | 0.175 | 94 | 0.274 | 95 |
|                | 30 | 0.0168 | 87 | 0.168 | 90 | 0.258 | 90 |
|                | 58 | 0.0157 | 81 | 0.159 | 85 | 0.244 | 85 |
|                | 90 | 0.0157 | 81 | 0.185 | 99 | 0.282 | 98 |
|                | 120 | 0.0161 | 83 | 0.175 | 93 | 0.267 | 93 |
| High level spike | 6\(^d\) | 0.0649 | 100 | 0.462 | 100 | 0.730 | 100 |
|                | 15 | 0.0642 | 99 | 0.449 | 97 | 0.711 | 97 |
|                | 20 | 0.0638 | 98 | 0.435 | 94 | 0.690 | 95 |
|                | 30 | 0.0549 | 85 | 0.400 | 87 | 0.631 | 86 |
|                | 58 | 0.0708 | 109 | 0.501 | 108 | 0.792 | 109 |
|                | 90 | 0.0549 | 85 | 0.449 | 97 | 0.701 | 96 |
|                | 120 | 0.0577 | 89 | 0.458 | 99 | 0.717 | 98 |

\(^a\)Analytical results unadjusted for recovery. % Remaining was calculated taking the mean analytical value at the initial timing as 100%.

\(^b\)“Total” refers to the sum of fosetyl-Al and phosphonic acid expressed as fosetyl-Al and was calculated by summing the concentration of fosetyl-Al and 1.44 times the concentration of phosphonic acid.

\(^c\)Results are expressed with one extra digit in order to calculate the percentage remaining. All analytical results of fosetyl-Al and some analytical values of phosphonic acid at the low-level spike were below the proposed LOQ but they were above the calculated LOQ (10 times the SD of analysis of freshly spiked wheat). Therefore, the values were put in parentheses and included in the above table.

\(^d\)Mean of 10 samples. The mean analytical values at the initial interval were taken from those in Table 3 (analysis of spiked samples at the low and medium levels 2 days after spiking and those at the high level 6 days after spiking).
Table 6. Statistical analysis of multiple-laboratory validation

|                      | Fosetyl-Al | Phosphonic acid | Fosetyl-Al | Phosphonic acid | Fosetyl-Al | Phosphonic acid |
|----------------------|------------|-----------------|------------|-----------------|------------|-----------------|
| Mean concn at the    | 0.006      | 0.053           | 0.019      | 0.187           | 0.065      | 0.462           |
| spike, mg/kg,\(^b\)  |            |                 |            |                 |            |                 |

Analytical results

|                      | 14         | 12              | 12         | 12              | 14         | 12              |
| Number of valid      |            |                 |            |                 |            |                 |
| analytical results   | (among 7 × 2 results) |             |            |                 |            |                 |
| Mean concn, mg/kg    | 0.006      | 0.052           | 0.019      | 0.176           | 0.055      | 0.426           |
| RSD, %               | 20.0       | 7.5             | 4.8        | 6.5             | 19.8       | 10.6            |
| Reproducibility      |            |                 |            |                 |            |                 |
| (RSD\(_R\), %)       | 33.8       | 12.7            | 23.2       | 5.9             | 24.0       | 9.8             |
| HorRat value         | 1.54       | 0.58            | 1.05       | 0.29            | 1.09       | 0.54            |

\(^a\)Analytical results unadjusted for recovery.

\(^b\) The “Mean concentration at the spike” refers to the analytical results shown in Table 3 (analysis of the spiked samples at the low and medium levels 2 days after spiking and those at the high level 6 days after spiking).
Table 7. Single laboratory verification of the method on brown rice (var. Nikomaru) spiked at two different levels\textsuperscript{a}

| Spike, mg/kg | Fosetyl-Al | Phosphonic acid | Fosetyl-Al | Phosphonic acid |
|-------------|------------|----------------|------------|----------------|
|             | 0.01       | 0.05           | 0.1        | 0.5            |
| Analytical results |           |                |            |                |
| Mean recovery, % | 82.9       | 88.0           | 88.3       | 82.6           |
| RSD, %      | 8.9        | 8.2            | 5.5        | 5.3            |
| RSD\textsubscript{i}, %\textsuperscript{b} | 9.1        | 9.7            | 6.8        | 5.3            |

\textsuperscript{a}The percent recoveries were calculated after subtracting the blank value (fosetyl-Al, 0.0000 mg/kg; and phosphonic acid, 0.0073 mg/kg).

\textsuperscript{b}Analysed in duplicates on each of 5 consecutive working days.
Table 8. Fosetyl-Al and phosphonic acid in samples of rice, wheat, six-row barley, hull-less barley grains

| Cereal grain | Grown in: | No. of samples | Fosetyl-Al | Phosphonic acid | Phosphonic acid in fosetyl-Al equivalents$^b$ |
|--------------|-----------|----------------|------------|----------------|---------------------------------------------|
| Certified organic produce | | | | | |
| Rice (brown) | Japan | 1 | No peak | (0.007) | (0.011) |
| Conventionally grown without using pesticides or fertilizers containing fosetyl-Al or phosphonic acid | | | | | |
| Wheat | Japan | 3 | No peak | (0.008-0.017) | (0.011-0.024) |
| Six-row barley | Japan | 1 | No peak | (0.019) | (0.028) |
| Hull-less barley | Japan | 1 | No peak | (0.019) | (0.028) |
| Conventionally grown (no information on the use of fosetyl-Al or phosphonic acid) | | | | | |
| Rice (Brown) | Japan | 10 | No peak | (0.007-0.010) | (0.010-0.014) |
| Imported | | | | | |
| Rice (white) | Thailand | 10 | No peak | (0.033)-0.199 | (0.047)-0.287 |
| Rice (white) | USA | 5 | No peak | (0.015-0.021) | (0.021-0.031) |
| Wheat | USA | 10 | No peak | (0.015)-0.178 | (0.022)-0.257 |

$^a$ Unadjusted for recovery. No peaks of fosetyl were observed in any of the samples. As the peak of phosphoric acid was observed on the chromatograph of every sample, the value shown on the chromatogram were included in the table in parentheses although they were below the proposed LOQ for phosphonic acid.

$^b$ Calculated by multiplying the concentration of phosphonic acid by 1.44.
Standard solution (containing fosetyl-Al equivalent to 0.01 mg/kg in grain) Fosetyl monitored at m/z 109→63

Brown rice sample Fosetyl monitored at m/z 109→63

Standard solution (containing phosphonic acid equivalent to 0.05 mg/kg in grain) Phosphonic acid monitored at m/z 81→79
Figure 1. MRM chromatograms of fosetyl and phosphonic acid in the standard solution and brown rice sample.