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Application Note

# Determination of Phosphonic Acid in Almonds, Apples, Rice, and Rooibos Using the QUPPe Method and LC-MS/MS

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## Abstract

Polar pesticides are widely applied worldwide in a variety of fields, including agriculture and public health, but pose significant analytical challenges due to the specific physicochemical properties of these compounds. Particularly problematic is phosphonic acid, whose small, low molecular weight and high polarity severely hinder its retention in reversed-phase liquid chromatography and its unequivocal identification via mass spectrometry detection. Thus, a reliable and efficient methodology able to overcome those problems is needed. This application note describes a method for the determination of phosphonic acid in plant-based commodities, based on the QuPPe extraction method, chromatographic separation with an ACQUITY™ Premier LC System and Anionic Polar Pesticide column, and final MS/MS measurement with a Xevo™ TQ Absolute Tandem Quadrupole Mass Spectrometer, acquiring two MRM transitions. Validation experiments were carried out in four different matrices (apple, rooibos, rice, and almond) at two levels of concentration (0.1 and 1 mg/kg) yielding relative recoveries between 77% and 107% with RSDs below 5% in all cases. This method was found to be accurate, sensitive, and specific for the determination of phosphonic acid residues at concentrations well below the regulatory limits.

## Benefits

- Retention greater than two column void volumes and separation from background interferences of phosphonic acid using HILIC chromatography
- Reliable identification and determination of phosphonic acid in different plant-based commodities, with lower method LOQs than current maximum residue limits (MRL)
- Method exceeds all criteria for method validation outlined in SANTE/11312/2012v2

## Introduction

Phosphonic acid is a highly polar organophosphonate compound that is commonly found in plant-based commodities at varying levels of concentration. Most organophosphonates may come from very different sources, are ubiquitously found in natural samples as byproducts of biogeochemical processes and, due to the chemical stability of the P-C bond, can ultimately be found in plant-based food and feed. In addition, synthetic organophosphonates such as glyphosate, fosetyl-Al or ethephon are commonly used in agriculture and may degrade into phosphonic acid. Finally, phosphonic acid itself may also be used as a fungicide in agrochemical applications.[1]

Due to its high polarity and low molecular mass, the liquid chromatography tandem mass spectrometry (LC-MS/MS) determination of this pesticide is especially challenging, since it shows insufficient interaction with typical reversed-phase liquid chromatography stationary phases. Additionally, the mass spectrometric detection is troublesome due to the low specificity of the ions selected for its measurement, as they can be subject to more interferences and background noise in LC-MS/MS methodologies. The European Union Reference Laboratory for Single Residue Methods (EURL-SRM) has published a compilation of single residue methods for a range of highly polar pesticides, including phosphonic acid.[2] Those methods are usually based on either ion chromatography (IC), hydrophilic interaction liquid chromatography (HILIC) or separation by mixed-mode columns. In this regard, the Anionic Polar Pesticide (APP) column is proposed as an alternative option for their determination, combining HILIC and IC interactions in diethylamine (DEA) substituted ethylene bridged hybrid (BEH) particles[3] allowing optimal retention and peak shape.

Recent application notes showed the analysis of anionic polar compounds using this approach, although, either they did not include phosphonic acid as an analyte[3, 4] or they are limited to high-water content matrices.[5]

In this application note, we propose a QuPPe-based method using an ACQUITY Premier System, an APP column and a Xevo TQ Absolute Tandem Quadrupole Mass Spectrometer for the determination of phosphonic acid in food of plant origin belonging to different crop groups: high-water content (apple), dry (rice), difficult (rooibos), and fat-containing (almond) commodities. The objective limit of quantification was set at 0.1 mg/kg which is well below the MRL established by the European Food Safety Authority (EFSA) for those products (with Commission Regulation (EU) 2024/2619 stating MRLs for almonds, 1000 mg/kg, apples, 70 mg/kg and rice, 3 mg/kg.

## Experimental

### Sample Preparation

All plant-based commodities were blended and homogenized in the presence of dry ice prior to analysis. Extraction was carried out by means of the general QuPPe-PO-Method from the EURL-SRM[2] with some modifications. The procedure applied is depicted in Figure 1.

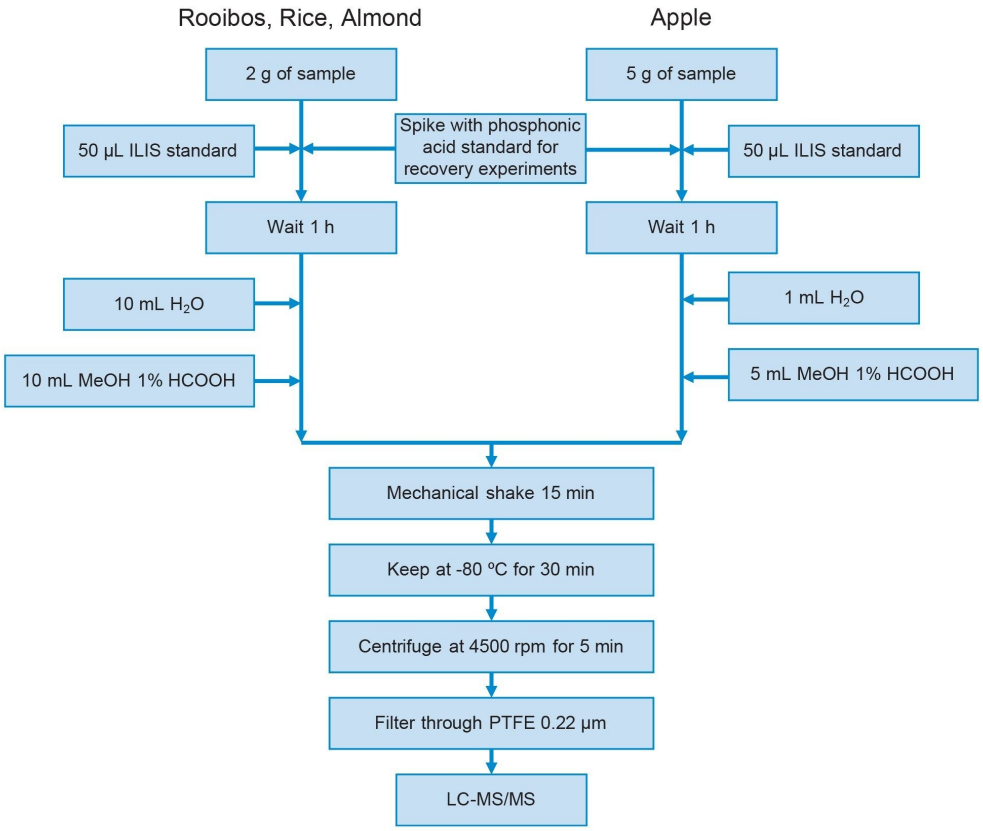


*Figure 1. Procedure for sample extraction prior to LC-MS/MS analysis.*

## LC Conditions

| | |
|---|---|
| LC system: | ACQUITY Premier System with QSM and FTN |
| Column: | Anionic Polar Pesticide 2.1 x 100 mm, 5 µm Column (p/n: 186009287) |
| Column temperature: | 40 °C |
| Sample temperature: | 10 °C |

| | |
|---|---|
| Injection volume: | 30 µL |
| Mobile phase A: | 10 mM ammonium acetate / 0.1% formic acid in HPLC water |
| Mobile phase B: | 0.1% formic acid in LC-MS grade methanol |
| Divert valve: | 0–2.5 minutes to waste |

## Gradient Table

| Time (min) | Flow (mL/min) | %A | %B | Curve |
|---|---|---|---|---|
| Initial | 0.5 | 10 | 90 | – |
| 1 | 0.5 | 10 | 90 | 6 |
| 3 | 0.5 | 85 | 15 | 2 |
| 6 | 0.5 | 85 | 15 | 6 |
| 8 | 0.5 | 10 | 90 | 1 |

## MS Conditions

| | |
|---|---|
| MS system: | Xevo TQ Absolute |
| Ionization mode: | ESI - |
| Capillary voltage: | 0.5 kV |
| Source temperature: | 150 °C |
| Desolvation temperature: | 650 °C |

| | |
|---|---|
| Desolvation gas flow: | 1000 L/h |
| Cone gas flow: | 150 L/h |
| Collision gas flow: | 0.15 mL/min |

## MRM Method

| Compound | Transition* | CV (V) | CE (eV) | Dwell time (s) |
|---|---|---|---|---|
| Phosphonic acid | **80.9>78.8 (Q)** | 10 | 15 | 0.08 |
| | 80.9>62.9 (q) | 10 | 15 | 0.08 |
| $^{18}O_3$-Phosphonic acid (ILIS) | **86.9>84.8 (Q)** | 10 | 15 | 0.08 |
| | 86.9>66.9 (q) | 10 | 15 | 0.08 |

**(Q) Quantitative transition; (q) confirmatory transition*

## Data Management

| | |
|---|---|
| MS acquisition software: | MassLynx™ 4.2 Software |
| Processing software: | waters_connect™ for Quantitation Software |

# Results and Discussion

## Chromatography

Chromatographic separation of the analyte was achieved using the APP column in all the matrices tested achieving adequate retention in compliance with the requirements for chromatography listed in SANTE 11312/2021 v2[6]. Moreover, no coeluting interferent peaks were observed. While sensitivity was good and allowed quantification of phosphonic acid at the lowest fortification level (0.1 mg/kg in sample), peak shape and retention

times were slightly affected by the matrix (Figure 2), however the use of ILIS allows identification even though the retention time has shifted in the matrix.[6] Despite those facts, integration and quantification was not negatively affected.

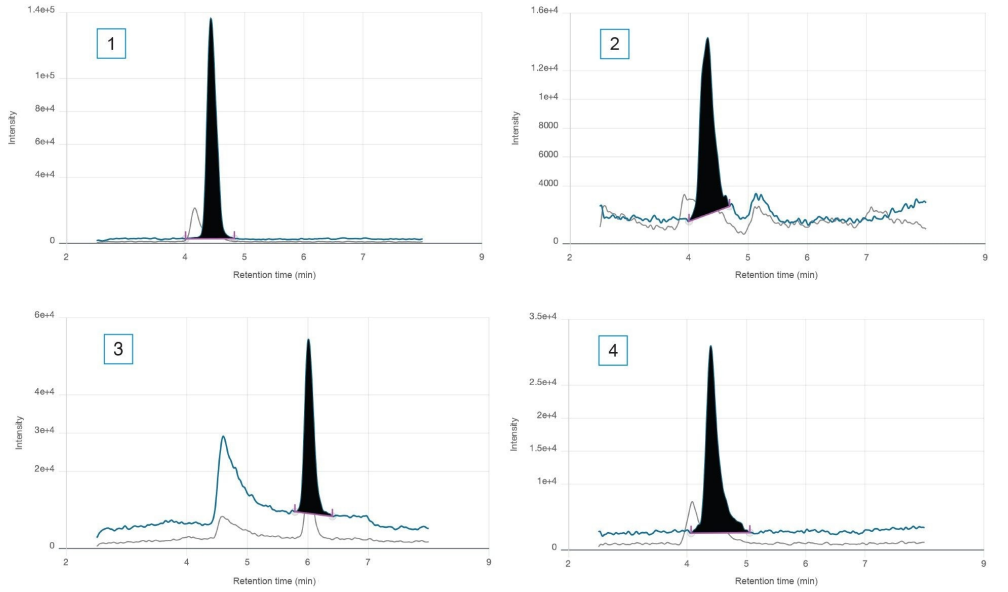


*Figure 2. Chromatograms corresponding to samples spiked at 0.1 mg/kg of apple (1), rooibos (2), rice (3), and almond (4).*

## Linearity

Matrix-matched calibration curves were used for quantification in the range of 25 ng/mL to 2500 ng/mL in vial, which corresponds to 0.1 mg/kg to 10 mg/kg in sample, with excellent linearity in all cases ($r^2$ >0.998) as illustrated in Figure 3.

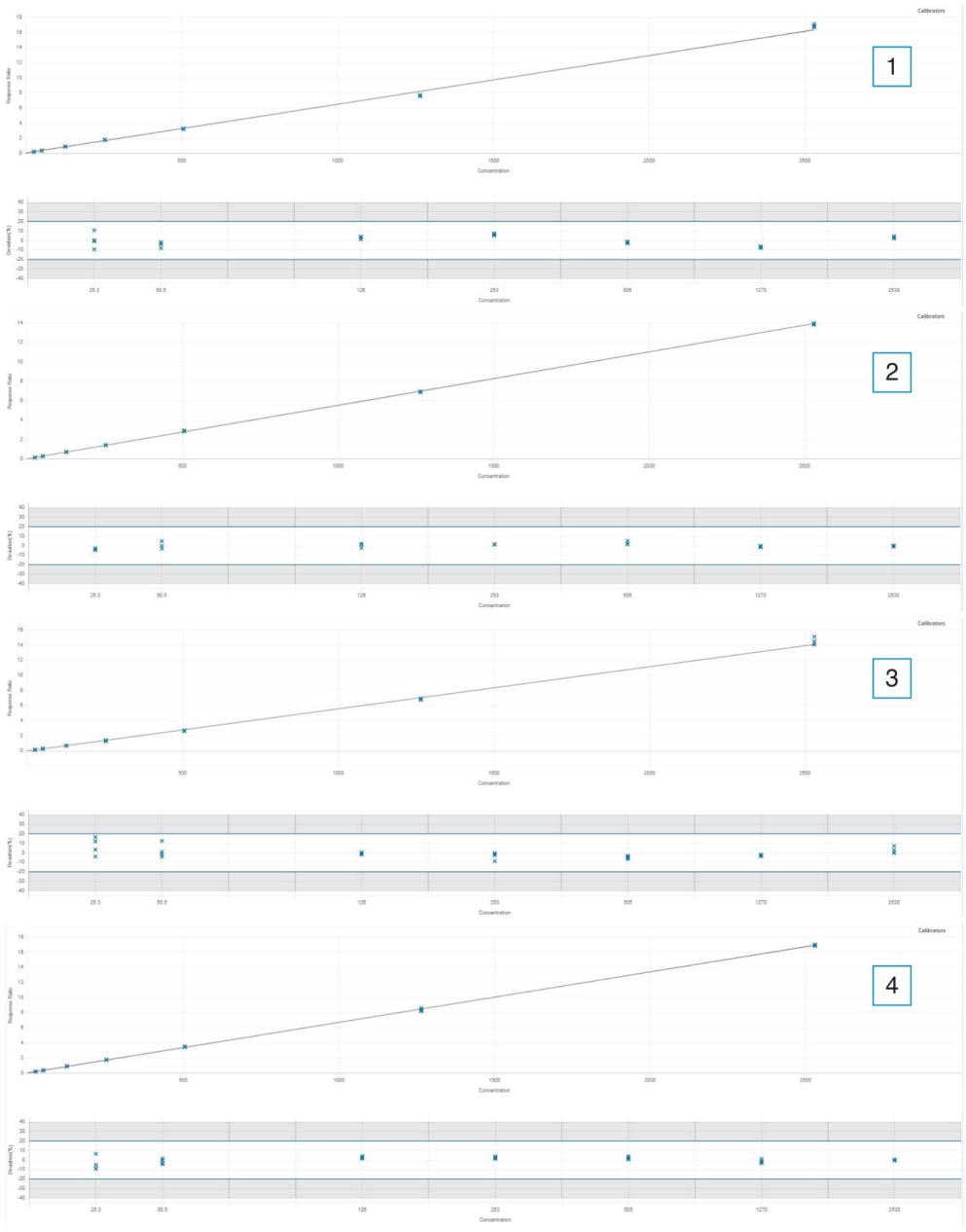


*Figure 3. Matrix-matched calibration curves of apple (1), rooibos (2), rice (3), and almond (4), between 25 ppb and 2500 ppb in vial corresponding to 0.1–10 mg/kg in sample.*

## Matrix effects

Although possible matrix effects related to signal suppression or enhancement were corrected by the use of matrix-matched calibration, $^{18}O_3$-phosphonic acid was also added as an isotopically labeled internal standard (ILIS) to both samples and calibration curves. Since the ILIS was added to samples before extraction, it acted as a surrogate, compensating for any potential errors associated with sample preparation as well as correcting any drifts in the analytical sequence. More so, the presence of the ILIS allowed confirmation of identity of the analyte when retention time differences were observed between matrices.

## Recovery and Limit of Quantification

Accuracy and precision were assessed by means of spiked samples at the target limit of quantification (LOQ) = 0.1 mg/kg and 10 x LOQ = 1 mg/kg. Five replicates were prepared for each matrix type and concentration level with satisfactory results in all cases as can be seen in Table 1 (results corresponding to the quantification transition):

| Spiked level | | 0.1 mg/kg | | 1 mg/kg | |
|---|---|---|---|---|---|
| Matrix | $r^2$ | %rec | RSD | %rec | RSD |
| Apple | 0.999993 | 77.5 | 2.2 | 94.3 | 1.6 |
| Rooibos | 0.999911 | 106.5 | 3.5 | 100.4 | 1.3 |
| Rice | 0.998711 | 107.1 | 4.6 | 90.1 | 3.7 |
| Almond | 0.999826 | 83.1 | 3.9 | 88.4 | 2.5 |

*Table 1. Linearity, recovery and precision of phosphonic acid in the four commodities.*

The correct identification of the compound in samples was achieved by co-elution of analyte and ILIS and compliance of the q/Q ratio deviation (≤30%) with regards to the average of the reference standards included in the calibration curve. Reliable quantification was supported by satisfactory accuracy and precision, with recoveries between 70–120% and RSD (%) ≤20%.

# Conclusion

The LC-MS/MS residue determination of phosphonic acid in several plant-based matrices from different commodity types has been described, achieving excellent accuracy, precision, selectivity, specificity, and linearity at concentrations well below the regulatory limits set by EFSA. The method is based on QuPPe extraction, chromatographic separation using the ACQUITY Premier LC System, the mixed-mode Anionic Polar Pesticides column and the Xevo TQ Absolute Tandem Quadrupole MS System operating in negative ion electrospray ionization mode. The method exceeded all criteria for method validation outlined in SANTE/11312/2012v2.

## References


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2. Anastassiades, M *et al.* Quick Method for the Analysis of Numerous Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC-MS/MS Measurement. 2023. *Food of Plant Origin* (QuPPe-PO-Method v12.2).
3. Simon Hird, Stuart Adams, Janitha De-Alwis. Evaluation of the Performance of a Method for the Determination of Highly Polar, Anionic Pesticides in Foodstuffs Using LC-MS/MS. Waters Application Note 720007505. 2022.
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6. Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed, SANTE 11312/2021 v2.


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