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Determination of Anionic Polar Pesticides in Spinach Using a Novel Application of Torus DEA Column Chemistry by Liquid Chromatography-Tandem Quadrupole Mass Spectrometry

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APPLICATION BENEFITS

Specific, targeted method for determination of a range of polar pesticides in spinach that is suitable for both official control and food business operators' due diligence testing offering:

- Improved chromatographic retention, peak shape, and selectivity compared to earlier methods.
- Sufficient sensitivity to determine residues at ppb levels in crude extracts without cleanup.

WATERS SOLUTIONS

Torus™ DEA Column

ACQUITY™ UPLC™ I-Class System

Xevo™ TQ-XS Mass Spectrometry

MassLynx™ MS Software

TargetLynx™ XS Application Manager

KEYWORDS

UPLC-MS/MS, anionic polar pesticides, glyphosate, QuPPe method

**Please note: This Application Note was developed on a Torus DEA Column, improved performance can be achieved using the Waters Anionic Polar Pesticide Column coupled with our most recent Application Notes please contact Waters Chemistry Technical Services with any questions www.waters.com/contact.*

INTRODUCTION

Pesticide residues resulting from the use of plant protection products on crops that are used for food or feed production may pose a risk factor for public health. A comprehensive legislative framework has been established in each country that defines rules for the approval of active substances, the use of plant protection products, and for the maximum amounts of residues permitted in food. Residue definitions are set during the evaluation process of the active substance, which may include relevant metabolites and other transformation products. Food surveillance testing programs check for compliance with maximum residue limits (MRLs) or tolerances, assess dietary exposure, and check for use of unauthorized pesticides. The food industry also undertakes testing of ingredients and finished products for due diligence, brand protection, or product release purposes. Some of the polar pesticides are among the most commonly used plant protection products so there is a need for robust methods to monitor food for residues to ensure compliance with local statutory maximum permitted limits. Where usage is approved, MRLs are often set relatively high (e.g. glyphosate in barley: 20 mg/kg[1] in the EU, 30 mg/kg in the U.S.[2]). Where no MRLs have been set the default MRL "at or about the limit of determination" applies. Although these tend to be higher than the 0.01 mg/kg set for most pesticides, they have been updated and reduced over last few years (0.3–0.1 mg/kg). Some polar pesticides have temporary MRLs or national action limits to facilitate trade.

Although various multi-residue LC-MS/MS methods are available to analyze food for pesticide residues, some pesticides are not amenable to such generic methods because they are highly polar and/or ionic in nature and so they are not extracted and/or show poor retention under the generic C_{18} conditions typically employed. Historically these compounds were treated as a series of selective single residue methods, adding significant costs, and so were often excluded from surveillance programs. The QuPPe (Quick Polar Pesticides) Method[3] allows the simultaneous extraction of many highly polar pesticides and their metabolites. QuPPe is typically used with LC-MS/MS instruments offering high sensitivity in order to deal with the significant matrix effects associated with the crude extracts (no clean-up).

Although QuPPe can be coupled with a plethora of chromatographic methods, including ion pair or derivatization with Reversed-Phase (RP), Porous Graphitized Carbon (PGC), Ion Chromatography (IC) options, Hydrophilic Interaction Chromatography (HILIC), and "mixed-mode" options, these methods have all been shown to have limitations. Problems have been reported that are associated with analytical scope (many different single residue methods needed), a need for specialized equipment (e.g. for IC), insufficient retention/selectivity, shifts in retention, significant ion suppression from matrix effects, and restrictions on operating backpressure curbing the flow rate that can be used and leaving the column susceptible to blockages.

Previously, we explored the strengths and weakness of some of these approaches for determining anionic polar pesticides in food.[4,5] In this application note we report the results of the validation of a method based upon QuPPE and the novel use of Waters® Torus DEA Column (p/n 186007616) as a UPLC Column for the cost-effective determination of a range of polar pesticides and their metabolites in spinach.

EXPERIMENTAL

Sample preparation and extraction

Samples of organic spinach were purchased from retail outlets. Spinach was stored frozen and homogenized in a blender. Test portions were extracted using the EURL Quick Polar Pesticides (QuPPe) method.[3] The details of the method are summarized in Figure 1.

The performance of the method was assessed using SANTE guidelines.[6] To assess the accuracy of the method, a number of test portions of spinach were spiked at 0.01 and 0.05 mg/kg (n=5). Solutions of matrix-matched standards were prepared over the range 0.0075 to 0.200 mg/kg (7.5 to 200 ppb) to determine the concentration of the anionic pesticides and metabolites in the spikes (using bracketed calibration).

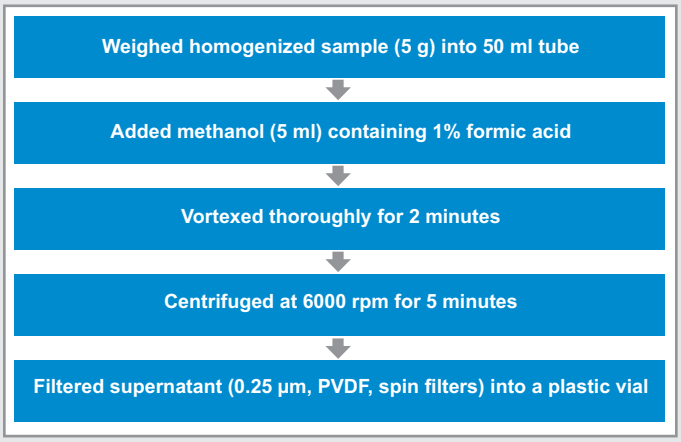

Figure 1. Schematic showing the Quick Polar Pesticides (QuPPe) method.

UPLC conditions

Before use, the LC system and column require simple cleaning and conditioning steps to remove metal ions that have been shown to interact with polar pesticides and cause poor peak shapes. Details can be found in the Start-up Guide.[7]

UPLC system:	ACQUITY UPLC I-Class with FL Sample Manager equipped with a 50-µL loop
Column:	Torus DEA 130Å, 1.7 µm, 2.1 mm x 100 mm
Mobile phase A:	50 mM Ammonium formate + 0.9% formic acid
Mobile phase B:	Acetonitrile + 0.9% formic acid
Flow rate:	0.5 mL/min
Injection volume:	10 µL
Injection mode:	Partial Loop Needle Overfill (PLNO)
Weak wash solvent:	90:10 Acetonitrile:water
Strong wash solvent:	10:90 Acetonitrile:water
Column temp.:	50 °C
Sample temp.:	10 °C
Run time:	16 min

Gradient*:

Time (min)	%A	%B	Curve
0.00	10	90	—
4.50	60	40	2
6.50	60	40	6
16.0	10	90	1

**Gradient start after injection with 320 µL delay.*

MS conditions

MS system:	Xevo TQ-XS
Ionization mode:	ESI-
Capillary voltage:	2.5 kV
Ion counting threshold:	40
Desolvation temp.:	600 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	150 °C
Cone gas flow:	300 L/Hr
Collision gas flow:	0.14 mL/min
Nebulizer gas pressure:	7 Bar

Data acquisition and processing

Data were acquired using MassLynx MS Software (v.4.2) and processed using TargetLynx XS Application Manager. The selection of MRM transitions and optimization of critical parameters was performed by infusion of individual solutions of all the analytes and evaluation of the data by IntelliStart™ Software, which automatically creates acquisition and processing methods. Soft ionization mode was enabled for ethephon. The optimum dwell time was set automatically using the Autodwell function.

*Table 1. MRM parameters for anionic polar pesticides (quantitative transitions in **bold**).*

Compound	Retention time (min)	MRM	Cone (V)	CE (eV)	Dwell time (s)
Perchlorate	1.72	**99>83** 99>67	20 20	18 45	0.085 0.085
Aminomethyl phosphonic acid (AMPA)	1.88	**110>63** 110>79	15 15	15 15	0.085 0.085
3-Methylphosphinico-propionic acid (MPPA)	2.33	**151>133** 151>107	15 15	12 16	0.031 0.031
Glufosinate	2.41	**180>85** 180>95	15 15	25 15	0.025 0.025
Chlorate	2.73	**83>67** 83>51	15 15	14 15	0.037 0.037
2-Hydroxyethyl phosphonic acid (HEPA)	2.76	**125>79** 125>95	15 15	14 12	0.023 0.023
Fosetyl aluminium	2.83	**109>81** 109>63	15 15	10 16	0.019 0.019
Ethephon	2.84	**143>107** 143>79	15 15	8 13	0.032 0.032
N-Acetyl glufosinate (NAG)	2.90	**222>136** 222>69	20 20	20 14	0.037 0.037
Glyphosate	2.99	**168>63** 168>150	15 15	15 10	0.105 0.105
Phosphonic acid	3.35	**81>79** 81>63	15 15	10 15	0.217 0.217

RESULTS AND DISCUSSION

Within laboratory method validation should be conducted on representative commodities to provide evidence that a method is fit for the purpose for which it is to be used. To meet the requirements of the SANTE guidelines this method has been tested on spinach, as a representative of commodities with high water, to assess sensitivity, mean recovery (as a measure of trueness or bias), precision (as repeatability RSDr), and the method Limit Of Quantification (LOQ). The LOQ is defined in the document as the lowest spike level meeting the method performance criteria for trueness and precision.

SENSITIVITY

Excellent sensitivity and selectivity was demonstrated from the analysis of matrix-matched standards. Figure 2 shows the response for the analytes at 0.01 mg/kg. The chromatographic separation of critical isobaric pairs is further highlighted in Figure 3. AMPA and fosetyl both generate ions that can prove to be isobaric and phosphonic acid can be formed due to degradation of fosetyl aluminium, hence the need for chromatographic separation.

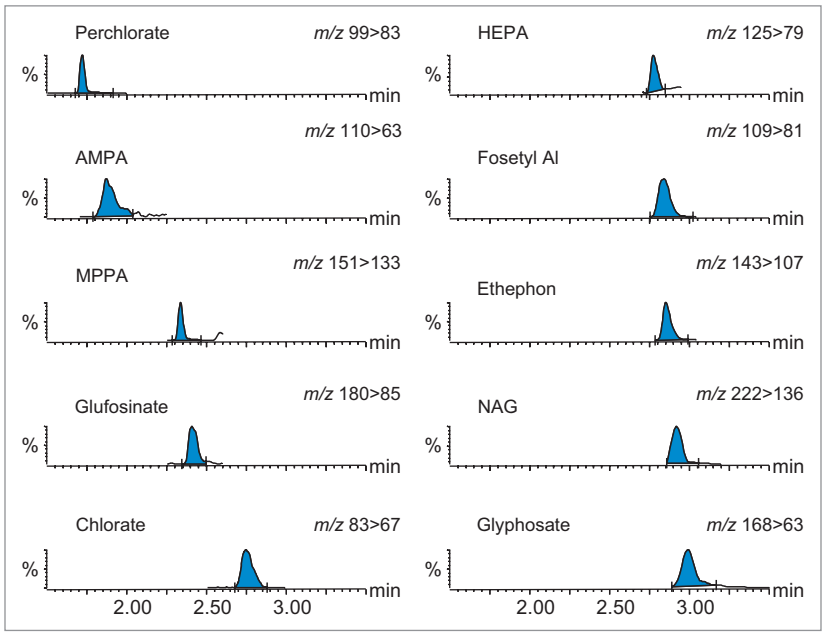

Figure 2. Typical chromatograms showing anionic polar pesticides from analysis of the matrix-matched standard at 0.01 mg/kg (10 ppb) in spinach.

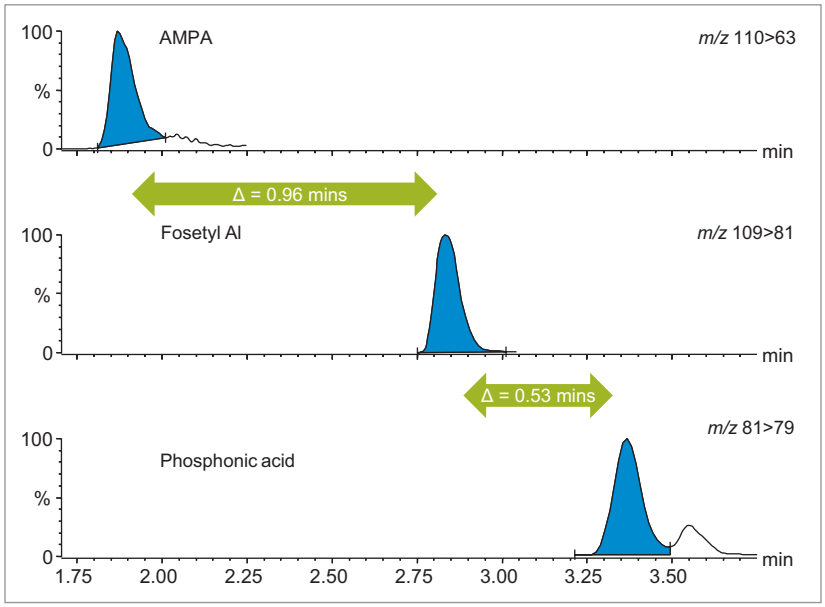

Figure 3. Typical chromatograms showing chromatographic separation of critical isobaric pairs from analysis of matrix-matched standard at 0.1 mg/kg (100 ppb) in spinach.

QUANTIFICATION

The linearity of response for the pesticides of interest was evaluated using bracketed calibration over a suitable concentration range, as shown in Figure 4. The coefficients of determination and the residuals (now referred to in the SANTE document as back-calculated concentrations) were excellent (r^2>0.99 and residuals <20%), demonstrating good repeatability of the measurements, in the absence of internal standards.

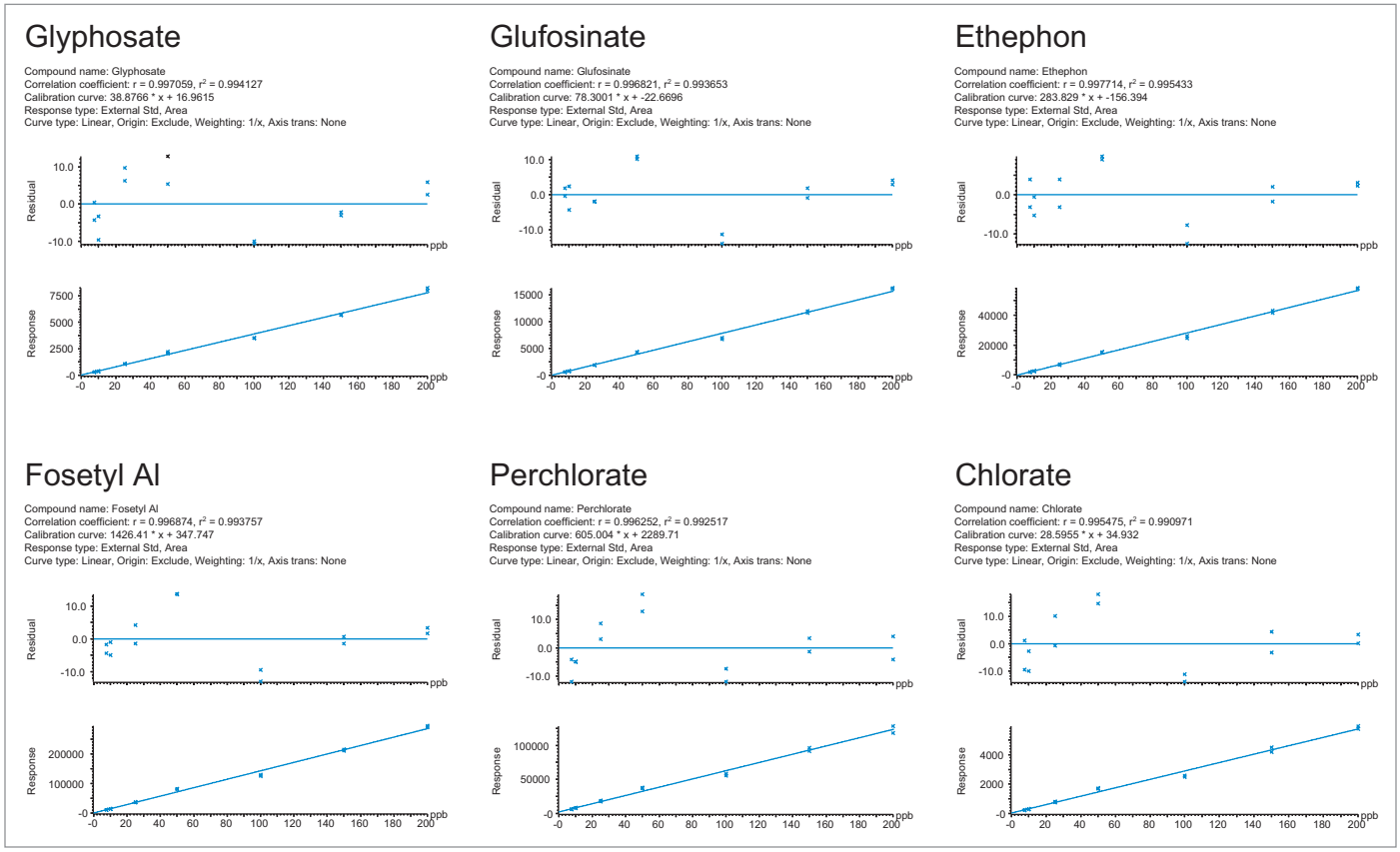

Figure 4. Calibration graphs for a selection of anionic polar pesticides, over the range in 0.0075 to 0.20 mg/kg (7.5–200 ppb), in spinach.

ACCURACY (TRUENESS AND PRECISION)

Five replicates were prepared at the targeted LOQ of the method but unfortunately data from one replicate at 0.05 mg/kg had to be excluded due to a preparation error. Phosphonic acid was detected in the blank used for preparation of the spikes so no calculation of trueness was possible. The concentration of phosphonic acid, detected in the blank, was calculated using standard addition (0.16 mg/kg).

Validation of the method demonstrated excellent performance for the quantification of anionic polar pesticides in spinach. Accuracy of the method at 0.01 and 0.05 mg/kg was excellent (Tables 2 and 3). Precision was good with repeatability (RSDr) between 1.1 and 9.7%. The recovery of glyphosate and perchlorate at 0.01 mg/kg would be improved by the use of internal standards. The mean recoveries from the spikes at 0.01 mg/kg for the other compounds were excellent: within the range 94 to 117%. Ion ratio and retention times agreed well with the reference values derived from the matrix-matched standards and almost all were well within the required tolerances. Only the ion ratios at 0.01 mg/kg for AMPA and HEPA exceeded the tolerance, albeit marginally. Table 4 shows a summary of the LOQ for each pesticide in spinach obtained from the validation of the method and the associated MRLs. The LOQ is defined in the SANTE document as the lowest spike level meeting the method performance criteria for trueness and precision. The target LOQ of 0.01 mg/kg was achieved for almost all of the analytes. An LOQ of 0.05 mg/kg was established for AMPA and HEPA purely due to marginal ion ratio failure.

Table 2. A summary of method validation for the determination of anionic polar pesticides in spinach spiked at 0.01 mg/kg.

Parameter	RT (min)		Ion ratio		Residuals	Recovery	RSDr
Criteria	**Range**	**±0.1**	**Range**	**±30%**	**≤20%**	**70–120%**	**≤20%**
Glyphosate	2.99-3.00	2.89-3.09	0.29-0.41	0.26-0.48	<13%	77%	3.3%
AMPA	1.86-1.90	1.77-1.97	0.54-0.80	0.43-0.79	<15%	102%	9.0%
Glufosinate	2.40-2.42	2.31-2.51	0.85-1.16	0.68-1.26	<14%	113%	6.0%
NAG	2.90-2.90	2.82-3.02	0.55-0.65	0.41-0.76	<20%	94%	4.1%
MPPA	2.33-2.33	2.23-2.43	0.56-0.62	0.41-0.76	<14%	117%	1.4%
Ethephon	2.84-2.84	2.75-2.95	0.23-0.29	0.23-0.42	<13%	106%	2.5%
HEPA	2.76-2.77	2.67-2.87	0.27-0.40	0.34-0.63	<14%	110%	2.1%
Fosetyl Al	2.83-2.83	2.73-2.93	0.12-0.14	0.09-0.16	<14%	105%	2.8%
Phosphonic acid	3.35-3.37	3.26-3.46	0.10-0.10	0.08-0.14	N/A	N/A	7.5%
Chlorate	2.73-2.74	2.64-2.84	0.05-0.07	0.04-0.07	<18%	101%	9.7%
Perchlorate	1.72-1.72	1.62-1.82	0.04-0.05	0.03-0.06	<19%	72%	5.9%

Table 3. A summary of method validation for the determination of anionic polar pesticides in spinach spiked at 0.05 mg/kg.

Parameter	RT (min)		Ion ratio		Residuals	Recovery	RSDr
Criteria	**Range**	**±0.1**	**Range**	**±30%**	**≤20%**	**70–120%**	**≤20%**
Glyphosate	2.99-3.00	2.89-3.09	0.38-0.40	0.26-0.48	<13%	101%	2.7%
AMPA	1.87-1.88	1.77-1.97	0.56-0.72	0.43-0.79	<15%	94%	1.8%
Glufosinate	2.40-2.43	2.31-2.51	0.89-1.06	0.68-1.26	<14%	106%	1.2%
NAG	2.90-2.94	2.82-3.02	0.58-0.62	0.41-0.76	<20%	100%	3.4%
MPPA	2.33-2.34	2.23-2.43	0.56-0.59	0.41-0.76	<14%	111%	3.3%
Ethephon	2.84-2.87	2.75-2.95	0.29-0.33	0.23-0.42	<13%	93%	1.1%
HEPA	2.76-2.79	2.67-2.87	0.39-0.46	0.34-0.63	<14%	98%	2.2%
Fosetyl Al	2.83-2.86	2.73-2.93	0.12-0.13	0.09-0.16	<14%	103%	1.2%
Phosphonic acid	3.35-3.39	3.26-3.46	0.09-0.11	0.08-0.14	N/A	N/A	7.3%
Chlorate	2.73-2.78	2.64-2.84	0.05-0.07	0.04-0.07	<18%	106%	2.5%
Perchlorate	1.72-1.76	1.62-1.82	0.05-0.05	0.03-0.06	<19%	90%	1.5%

Table 4. Summary of values for LOQ from method validation for the determination of anionic polar pesticides in spinach and associated MRLs.

Parameter	LOQ (mg/kg)	EU MRL (mg/kg)
Glyphosate	0.01	0.1
AMPA	0.05	None
Glufosinate	0.01	0.06
NAG	0.01	Part of glufosinate
MPPA	0.01	Part of glufosinate
Ethephon	0.01	0.05
HEPA	0.05	None
Fosetyl aluminium	0.01	75
Phosphonic acid	N/A	Part of fosetyl aluminium
Chlorate	0.01	None
Perchlorate	0.01	0.5

CONCLUSIONS

The use of the Torus DEA Column represents a robust, cost-effective solution, providing excellent chromatographic performance for a wide range of anionic polar pesticides and metabolites. This is achieved in a single analysis, without being required to use derivatization, specialized equipment, or atypical mobile phases. This method can be easily implemented in routine testing laboratories as it is compatible with existing conventional HPLC and UPLC instrumentation. It has been demonstrated as suitable for checking compliance with EU MRLs and has the potential for screening at much lower concentrations, for example for food business operators' due diligence testing. Although we have shown validation data in spinach that meet SANTE criteria, scientists must fully validate the method on their commodities of interest within their own laboratories in order to demonstrate that, when coupled with their extraction protocols, the method is fit for purpose.

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