Application note | 000770

Food safety

Assessment of mixed-mode chromatography for underivatized glyphosate and glufosinate analysis in foodstuffs

Authors

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Keywords

Acclaim Trinity P1 column, glyphosate, glufosinate, anionic polar pesticides, QuPPe, Vanquish Flex, mixed mode

Goal

To develop an LC method based on the Thermo Scientific™ Acclaim™ Trinity P1 column to separate and quantify glyphosate, glufosinate and its metabolites in food samples

Application benefits

Simplification of the analytical process by preventing compound derivatization and eluent suppression

Introduction

N-(phosphonomethyl)glycine, also known as glyphosate, is an organophosphorus compound. Discovered in 1950, glyphosate was recognized for its herbicidal effectiveness in 1975 and since then has been widely used in agriculture and horticulture to improve cultivated crop yields. It is currently approved for use in Europe until December 15th 2022.[1] In 2017, glyphosate accounted for 34% of all herbicides used by EU countries.[2] Between 2013 and 2017, the glyphosate market totaled around 44,000 tons.[2] The results of this intensive use are glyphosate residues in food, which have been the subject of recent controversy. To control the presence of this herbicide, the European Commission defined the Maximum Residues Limit (MRLs) permitted. Significant MRL values for glyphosate are reported in Table 1 by product family.

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Table 1. MRLs for glyphosate, Commission Regulation (EU) No 293/2013

Product	Glyphosate MRL (mg/kg)
Apples	0.1
Cucumber	0.1
Onions	0.1
Tomatoes	0.1
Lamb's lettuce	0.1

Glufosinate is another efficient broad-spectrum herbicide used to control weeds and was an active substance used in Europe between 2007 and 2018. While it is not currently authorized in Europe, many third-world countries approve of its use. Even without an authorized European market, the global glufosinate market, valued at $545 million in 2018, is projected to reach $2,097.7 million by 2026[3]. MRLs for glufosinate have been expressed as the sum of glufosinate and its metabolites, 3-hydroxy(methyl)phosphonyl propionic acid (3-MPPA) and *N*-acetyl-glufosinate (NAG). Due to the large-scale use of these common herbicides, many products can be contaminated by active substances or metabolites. For this reason, a fast, simple, and specific analytical method is required to cope with the increasing number of potentially contaminated samples.

We developed a new approach based on two pillars to meet EU regulation expectations. The first is easy and fast: the LC method simplification without any derivatization, and the second is simply a short cycle time. This study describes an LC/MS method under the negative ion-spray ionization mode to directly determine glyphosate, glufosinate, and metabolites in various matrices.

Experimental

Reagent and consumables

- Methanol, Optima™ LC/MS Grade, Fisher Chemical™ **(P/N 10031094)**
- Acetonitrile, Optima™ LC/MS Grade, Fisher Chemical™ (**P/N 10489553**)
- Formic acid, Thermo Scientific™ Pierce™ LC-MS grade, 50 mL **(P/N 13454279)**
- Ammonium formate, Optima™ LC/MS grade, Fisher Chemical™ (P/N 11377490)
- Ultra Pure Water produced by Thermo Scientific™ Barnstead™ Smart2Pure™ Pro water purification **(Model Smart2pure Pro UV/UF 16LPH)**
- Fisherbrand™ 1 mL plastic syringe PP **(P/N 14955-456)**
- Thermo Scientific™ Titan3™ syringe filter, 17 mm PVDF membrane **(P/N 44513-PV)**
- 1.2 mL, 9 mm glass vials (P/N 1.2-UHRSV)
- Pre-slit PTFE vial caps (P/N 9-SCK(B)-ST1X)

LC-MS/MS setup

The detailed design used for this study is outlined below and in Figure 1.

- Thermo Scientific™ Vanquish™ Flex UHPLC system, modified and consisting of:
  - Thermo Scientific™ Vanquish™ Dual Pump F **(P/N VF-P32-A)**
  - Thermo Scientific™ Vanquish™ Split Sampler FT **(P/N VF-A10-A)**
  - Thermo Scientific™ Vanquish™ Column Compartment H **(P/N VH-C10-A)**
  - Thermo Scientific™ Dionex™ ICS-6000™ SP Analytical – Gradient with Degas **(P/N 22181-60001)**
  - Thermo Scientific™ Dionex™ GM-4 2 mm gradient mixer (P/N 049136)
- Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer **(TSQ02-10002)** equipped with the Thermo Scientific™ OptaMax™ Duet NG source housing (OPTON-32104)
- Thermo Scientific™ Dionex™ PEEK Viper™ assembly, 0.007 i.d., 9.0 in. (229 mm), CD (P/N 088835) x2
- Thermo Scientific™ Dionex™ PEEK Viper™ assembly, 0.007 i.d., 7.0 in. [178 mm], ED (P/N 088809) x2
- Thermo Scientific™ Dionex™ PEEK Viper™ loop, 25 µL, 0.007 i.d. (1007 mm) **(P/N 302893)**
- Thermo Scientific™ Dionex™ PEEK Viper™ loop, 2.5 µL, 0.007 i.d. (100 mm) **(P/N 302899)** x2
- Union tee, HPLC, PEEK, 1/16 in. orifice 0.020 in. thru-hole, 10–32 **(P/N P-727)**
- Thermo Scientific™ NanoViper™ 0.075 mm i.d. x 550 mm l, PEEK **(P/N 6041.5760)**

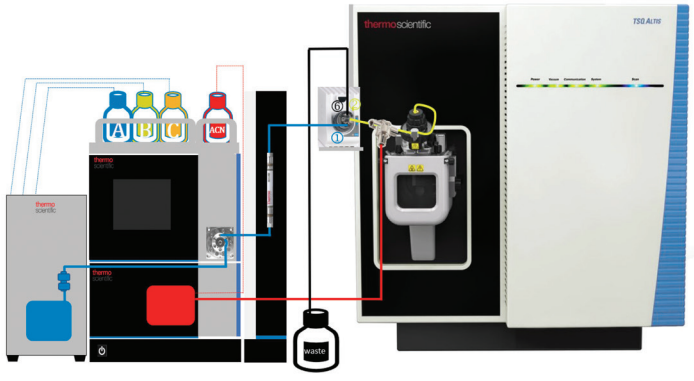


Figure 1. Optimized LC-MS/MS fluidics pathways: elution inert path (blue line) and make-up pathway (red line)

LC conditions

Table 2. LC conditions

LC column	Thermo Scientific™ Acclaim™ Trinity P1 100 x 2.1 mm, 3 µm, **P/N 071389**
Mobile phase A	100 mM Ammonium formate, pH = 3
Mobile phase B	Water
Mobile phase C	Acetonitrile
Elution flow rate	0.4 mL/min
Gradient	See Table 3
Column oven	40 ˚C Still air mode
Injection volume	15 µL
Sampler wash solution	Water 90 / methanol 10 (vol / vol)

Table 3. Gradient details

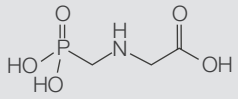
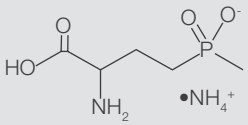
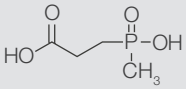
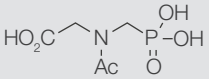
Time (min)	Flow rate (mL/min)	%A	%B	%C	%D
0	0.4	5	95	0	0
2	0.4	5	95	0	0
6.5	0.4	35	65	0	0
7	0.4	60	0	40	0
11	0.4	5	0	95	0
11	0.4	5	0	95	0
12	0.4	5	0	95	0
12	0.4	5	95	0	0
16	0.4	5	95	0	0

MS conditions

Table 4. MS parameters and settings

Run time	16 min
Ion source	H-ESI
Source positioning	Between M and L
Spray voltage	Negative mode, 1,000 V
Sheath gas	60
Auxiliary gas	15
Sweep gas	1
Ion transfer tube temperature	350 ˚C
Vaporizer temperature	400 ˚C
Make up	0.4 mL/min acetonitrile
Experiment type	Selected reaction monitoring (SRM)
Dwell time	10 ms
Chromatography peak width	7 s
Collision gas pressure	2.5 mTorr
Q1 resolution	0.7 FHMW
Q3 resolution	1.2 FHMW

Table 5. Chemical details and selected reaction monitoring (SRM) for each phosphated herbicide and metabolite

	Glyphosate	Glufosinate	3-MPPA	N-acetyl glufosinate
Chemical formula				
Retention time (min)	5.73	3.34	8.11	10.11
Ionization mode	*Negative*	*Negative*	*Negative*	*Negative*
Quan ion (CE)	168 → 150 (10)	180 → 63 (40)	151 → 63 (33)	222 → 180 (16)
Conf ion 1 (CE)	168 → 63 (20)	180 → 85 (20)	151 → 133 (8)	222 → 59 (15)
Conf ion 2 (CE)	168 → 124 (10)	180 → 136(20)	151 → 78 (31)	222 → 160 (15)
RF (V)	38	43	35	46
Source fragmentation (V)	0	0	0	0

Software

Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System was used for data acquisition and analysis.

Sample preparation

Commercial standard solutions were stored at -20 ˚C. One mix solution at 1 ppm was prepared for standard compounds using ultrapure water. Internal deuterated standard mix (ISTD) solution at 1 ppm was prepared with water and stored at -20 ˚C. Samples matrices were prepared using a slightly modified EURL QUPPE protocol,[4] by first weighing 10 g of fresh sample in a 50 mL polypropylene tube and then adding 10 mL of extraction solution (methanol with 1% formic acid). Sample homogenization was performed with a Fisherbrand™ 150 Homogenizer with a stainless steel probe for 5 minutes. The final volume was adjusted up to 20 mL with acidified methanol. A 50 mL tube was frozen more than 120 minutes at -20 ˚C, defrosted, and then centrifuged for 5 minutes at 7,000 rpm. An aliquot (1 mL) of the supernatant was withdrawn using a syringe, filtered through a 0.45 µm syringe filter, and diluted five times with water into the vial. 200 µL of matrix, 100 µL ISTD stock solution 1 ppm, 10 µL standard stock solution 1 ppm and 690 µL of water were added to the vial.

Results and discussion

Separation, detection, and confirmation

The Acclaim Trinity P1 column with the novel stationary phase offers stronger interaction with polar anionic compounds. This unique mixed-mode chemistry provides strong retention, allowing analytes to be eluted with complete resolution and limiting interactions with non-retained matrix compounds. Figure 3 illustrates the separation of each compound—glyphosate, glufosinate, 3-MPPA, and *N*-acetyl glufosinate. Three SRM were selected for each compound for quantitation and confirmation, and the respective retention times were 5.7, 3.3, 8.1, and 10.1. Using an inert pump involved a good peak shape for glyphosate, making the automated integration process easy.

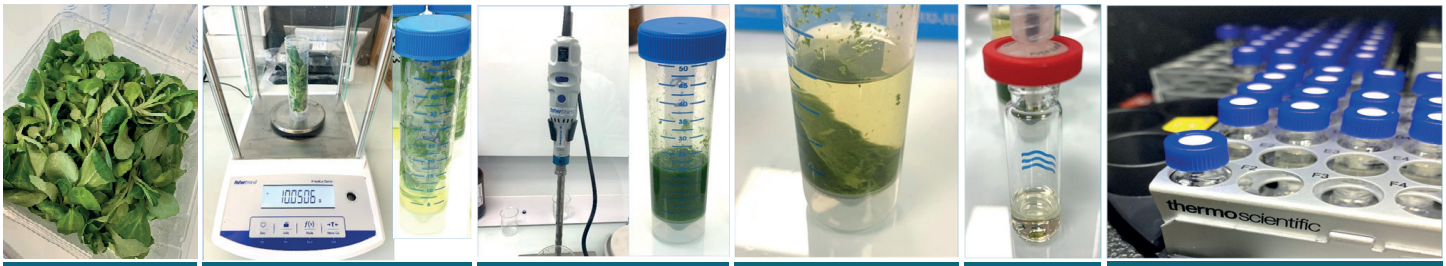


Figure 2. Sample preparation process

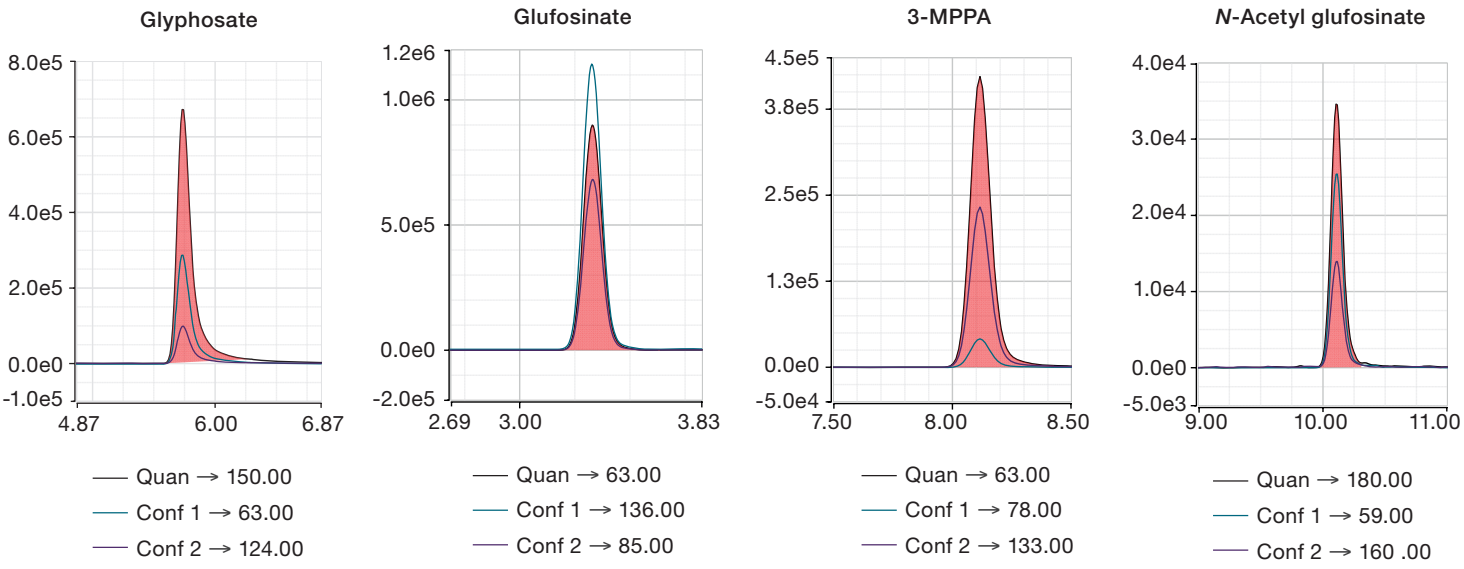


Figure 3. Direct MS component visualization in Chromeleon CDS 7.3 (100 ppb of standard mix solution)

Calibration

The insolvent calibration curves were created and ranged from 5 to 250 ppb (Figure 4). Calibration curves built using six levels were all based on the linear model. To anticipate the potential matrix effect on the MS response, each value was corrected using the corresponding labeled standard. Coefficients of determination were all above 0.996 (Table 6).

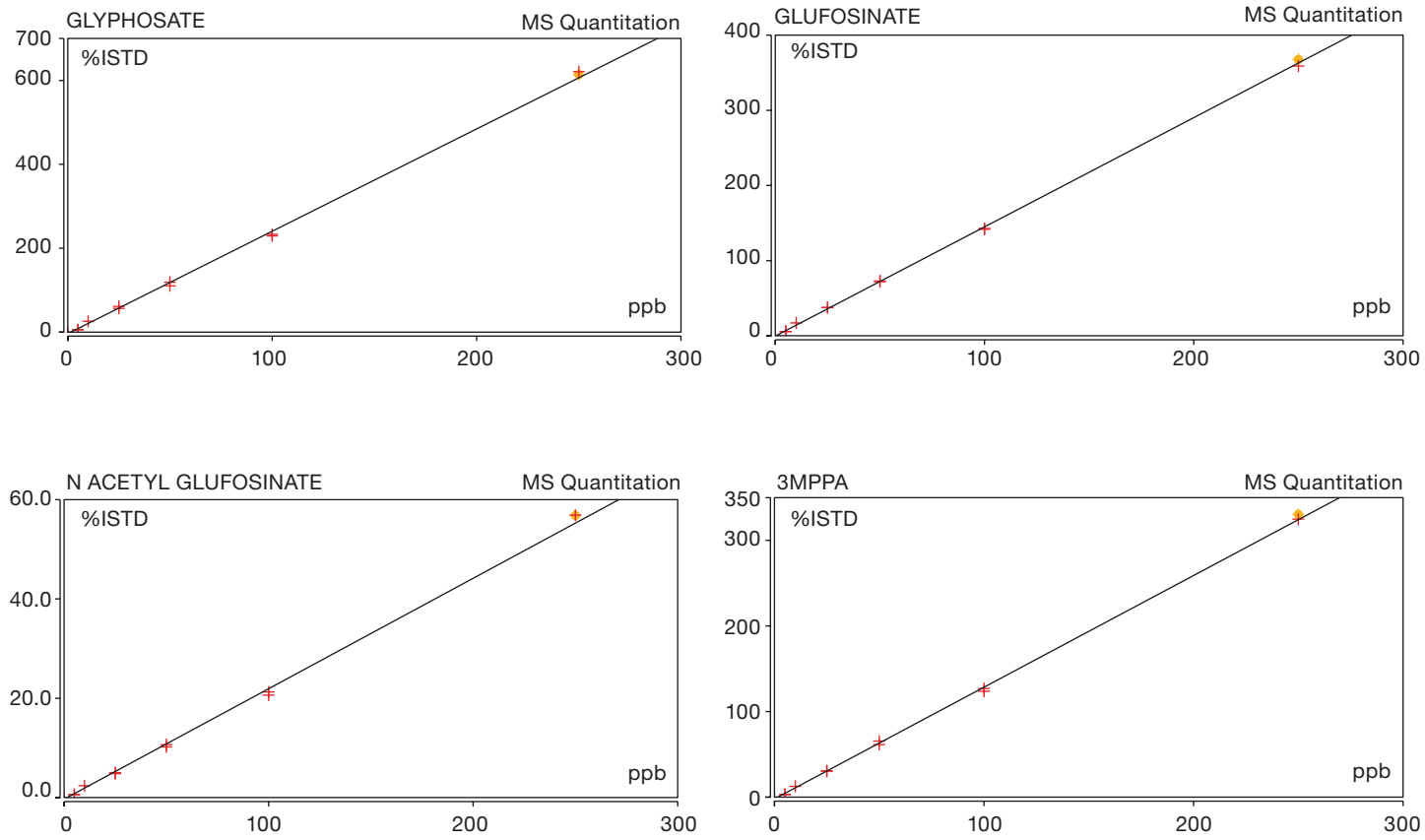


Figure 4. Calibration curves determined after a 15 µL injection of 5, 10, 25, 50, 100, and 250 ppb standard solution of glyphosate, glufosinate, 3-MPPA, and *N*-acetyl glufosinate. Each calibration curve was corrected using the respective labeled internal standard.

Table 6. Calibration information for each component: cal. type, weight, R^2, and labeled internal standard are used for data correction

Compound	Calibration type	Weight	R^2	ISTD
Glyphosate	Linear	1/X	0.99701	^{13}C ^{15}N glyphosate
Glufosinate	Linear	1/X	0.99867	D_3 glufosinate
3-MPPA	Linear	1/X	0.99793	D_3 3-MPPA
***N*-acetyl glufosinate**	Linear	1/X	0.99688	D_3 *N*-acetyl glufosinate

Method assessment in food matrices

Figure 5 shows the responses for glyphosate, glufosinate and its metabolites in each studied matrix: apple, tomato, onion, cucumber, and lamb's lettuce spiked at 10 ppb. The 10 ppb standard solutions were injected before the sample set to illustrate that signal-to-noise ratio is widely suitable for matrix sample investigation. Due to our sample preparation, 10 ppb detected in the vial corresponds to an MRL of 0.1 mg/kg for glyphosate or glufosinate. All matrices spiked at 10 ppb were injected, and component responses were drastically reduced by matrix interference, making internal calibration a must. The most impacted matrix in our sample set was lamb's lettuce, with a considerable signal reduction for *N*-acetyl glufosinate metabolite. Signal reduction is 51% and 67% for *N*-acetyl glufosinate in tomato and lamb's lettuce respectively. However, the amount of *N*-acetyl glufosinate was correctly determined using the internal standard for the spiked lamb's lettuce sample.

The proposed method in this study was assessed to analyze target analytes in different matrices. Figure 6A clearly illustrates the preliminary information for this new methodology: retention time stability was preserved over the sequence and seems independent of the matrix's type. The concise time window reduced false positives and allowed an automated integration process. Compounds detected were evaluated for area level. Figure 6B provides a graphical representation of areas, showing that *N*-acetyl glufosinate was weaker than the others.

On the other hand, area variability was due to the cross-impact of compound and matrix. For example, lamb's lettuce matrix negatively impacted the four spiked compounds at a minimum of fifty-five percent. In contrast, the apple matrix significantly affected glufosinate and not glyphosate, 3-MPPA, and *N*-acetyl glufosinate.Internal standard correction was critical to obtain accurate and expected results and compensate for the effect of the amount calculation. Including all results independently of the matrix, the relative standard deviation was lower than 7% for glyphosate, glufosinate, and 3-MPPA. For *N*-acetyl glufosinate, the relative standard deviation was increasingly impacted by the value obtained in the tomato matrix.

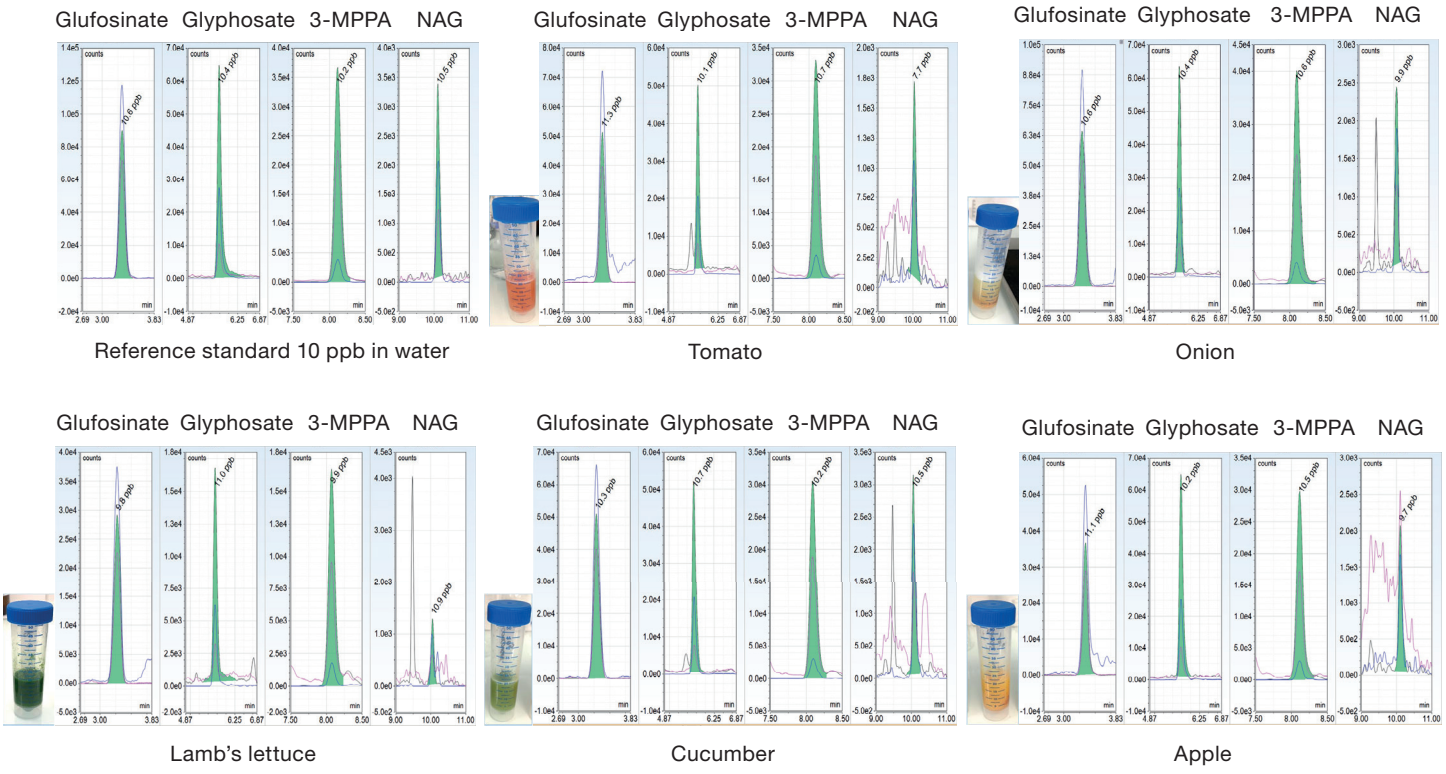


Figure 5. Direct MS component visualization in Chromeleon CDS 7.3 illustrating quantification and confirmation ion traces for each detected peak (10 ppb of standard mix solution or 10 ppb spiked matrices)

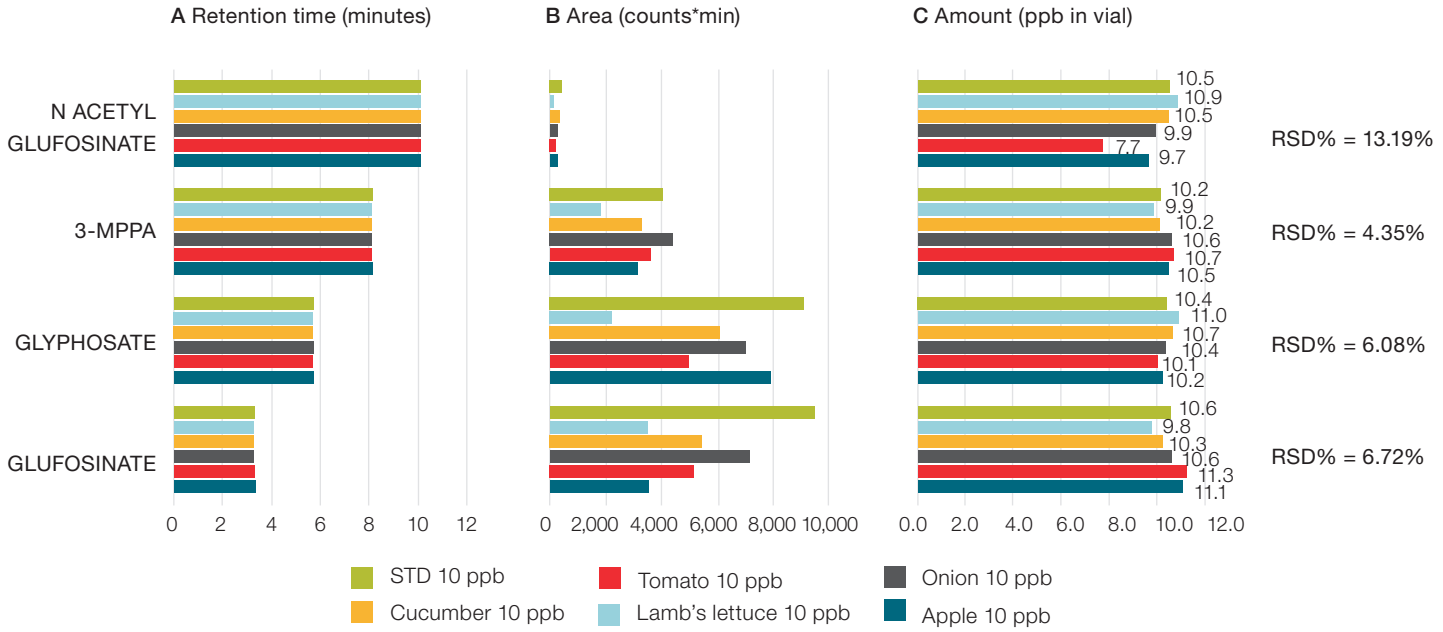


Figure 6. Graphical representation for each compound's retention time (A), area (B), and amount (C)

Conclusion

This new analytical LC/MS workflow based on the modified QuPPe method supports underivatized polar pesticides analysis in the various high-water content matrix. The LC-MS/MS method was developed using an Acclaim Trinity P1 mixed-mode column. The results showed that the sensitivity, linearity, and retention time precision aligns with EU MRLs.

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