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Milestone M7

Analytical Performances of prototype assays suitable for validation

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1. Introduction

The aim of WP2 – Task 2.6 is to develop and validate a rapid and fully automated assays for the detection of glyphosate in maize in order to i) provide a reliable and high throughput tool for on-site safety testing and ii) generate new occurrence data in a standardized format suitable for integration in the knowledge and data exchanging platform/infrastructure (Task 2.8)
Glyphosate (N-(phosphonomethyl)glycine), is an organophosphorus broad spectrum, non-selective, post-emergence herbicide. It is a chemical used worldwide in plant protection and its global consumption has increased over the years, the global glyphosate market worth $8 billion in 2022 is estimated to reach $10.6 billion by 2030, recording a compound annual growth rate (CAGR) of 3.6% for the period 2022-2030 [1].
Glyphosate has been thoroughly assessed by Member States, the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) in recent years due to its controversial toxicity. A step-by-step account of the renewal process can be found on the European Commission (EC) website [2]. The last update in the renewal process refers to the adoption of Implementing Regulation (EC) 2023/2660 by the Commission in November 2023 to renew the approval of glyphosate for 10 years. Glyphosate was approved even though neither the Standing Committee nor the Appeals Committee gave an opinion on the renewal proposal (i.e. there was no qualified majority for or against). This is consistent with EU legislation requiring the Commission to adopt an implementing regulation even without qualified majority, as in the case of glyphosate [3]. Maximum Residue Levels (MRLs) are set with a view to protect vulnerable groups, by taking into account toxicological safety thresholds as calculated in the toxicological assessment (Regulation (EC) No 396/2005) [4]. In the case of glyphosate, a revision of MRLs has been suggested, considering inclusion of metabolites of glyphosate [4,5]. For **enforcement purposes,** the "main residue definition" included the sum of glyphosate, AMPA and N-acetyl-glyphosate for plant with glyphosate tolerant genetically modified varieties currently available on the market, whereas glyphosate only can be considered a sufficient marker for enforcement in conventional crops. For **risk assessment,** a general residue definition covering both conventional and genetically modified crops was proposed as the sum of glyphosate, AMPA, N-acetyl-glyphosate and N-acetyl-AMPA, expressed as glyphosate. In the case of maize grains MRL was set to 1 mg/kg in the Regulation (EC) No 396/2005.
The assay developed in Task 2.6 is a lateral flow test, and specifically an immunochromatographic strip test (ICT) based on the indirect competition immunoassay.
The aim of the assay is to generate glyphosate contamination data allowing both the analysis of on-site commodities and facilitating, through automation, the management and collection of data in quality control laboratories. The data collected can be used to build models for risk prediction related to glyphosate contamination. In addition, the impact of glyphosate on production, consumers and the environment can be studied with the help of AI-based models capable of integrating residue occurrence data with different sources data (weather conditions, crop variety, soil type).

The present report describes the achievement of **Milestone M7**, i.e. to obtain a prototype assay showing analytical performances suitable for validation. The process comprised the following steps:

1) Setting up an optimized protocol for maize sample analysis
2) Building matrix matched calibration curves
3) Testing prototype stability (including testing of different production lots)
4) Evaluating sensibility and precision of the assay
5) Evaluating assay specificity (cross-reactivity study)
6) Setting up a confirmatory method to be used in the validation study

2. Results

2.1 Setting up an optimized protocol for maize sample analysis

2.1.1 Principle of test

Prototype strip tests were assembled at R&D labs of Vicam, a Waters business, then provided to CNR for testing and optimization of assay reagents and protocols for the project purposes.

The prototype is a lateral flow test, also called immunochromatographic strip test (ICT) and it is based on the indirect competition immunoassay approach.

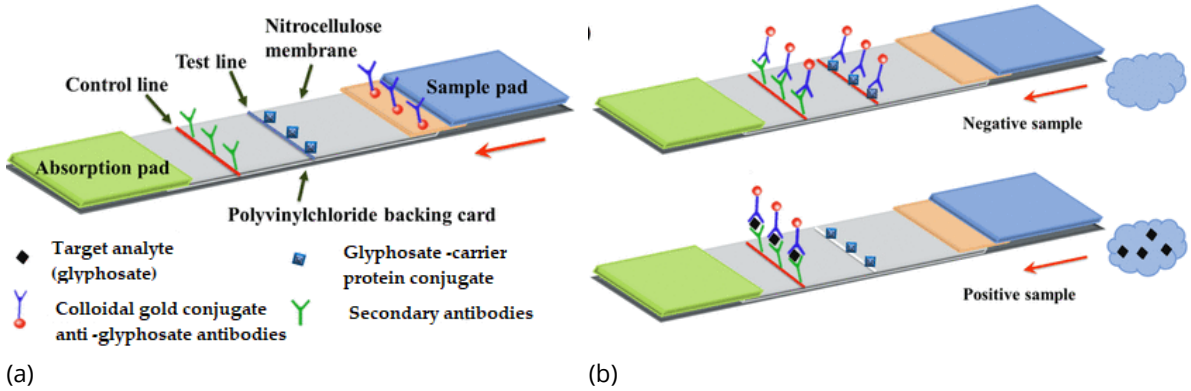

Figure 1: schematic illustration ofa typical lateral flow test strip (a) principle of the lateral flow strip test in negative sample and positive sample (b)

A typical lateral flow test strip (presented in Figure 1) consists of four overlapping membranes that are mounted on a backing card for better stability and handling: (1) the sample application pad, (2) the conjugate pad, (3) the nitrocellulose membrane, (4) the adsorbent pad.
The sample pad ensures the analyte contact and transport along the system, on the conjugate pad antibodies specific to the target analyte (for instance glyphosate) conjugated to colloidal gold particles were immobilized whereas in the nitrocellulose membrane were pre-immobilized antigens and secondary anti-species antibodies to form the test line and control line, respectively. Finally, the adsorbent pad controls the liquid's flow rate across the membrane and prevents sample backflow. After application of the sample solution (or sample extract) on the sample pad, the solution migrates by capillary action, and upon reaching the conjugate pad and dissolves colloidal gold-conjugate anti-glyphosate antibodies immobilized on the conjugate pad.
When target analyte is not present in the sample, the colloidal gold-conjugate anti-glyphosate antibodies migrate along the membrane and bind the analyte carrier protein conjugate on the test line. The excess of colloidal gold-conjugate anti-glyphosate antibodies also bind to the secondary antibodies on the control line. Therefore, in glyphosate-negative samples, two lines emerge on the strip.
When target analyte is present in the sample, the colloidal gold-conjugate anti-glyphosate antibodies on the conjugate pad binds it and limiting the amount of colloidal gold-conjugate anti-glyphosate that could bind to the coating analyte-carrier protein conjugate on the test line. Both

free colloidal gold-conjugate anti-glyphosate antibodies and conjugate with glyphosate are captured by to the secondary antibodies on the control line. Therefore, in positive samples, the T line has a weaker colour intensity than in negative sample. Consequently, the colour intensity of the T line is inversely proportional to the concentration of glyphosate in the sample. The C line should always emerge; otherwise, the procedure was incorrectly performed, or the strip was poorly assembled.
Quantitative analysis is performed by reading the optical intensity of the test line and control line with a portable photometric reader. The strip reader connects with a laptop, which has an installed software that transduces the line intensities into peak area. Peak areas are then converted in mass fractions by using a specific calibration curve.

2.1.2 Optimized protocol for sample preparation and assay analysis

The optimized protocol for maize analysis was show in figure 2
Three grams of maize are weighed into a test extraction tube with 30 ml of water and blended for 2 min. The sample is filtered and 1 ml (corresponding to 0.1 g of matrix) is transferred in 2 ml plastic test tube with cap. Then 0.1 ml of reagent A (buffer solution) and 0.1 mL of reagent B (derivatization solution) are added and the sample is vortexed for 15 seconds. The strip is left at room temperature for 5 min. 100 µl of the final sample are pipetted onto the lateral flow and allowed to run for 5 min, at room temperature. After running, the lateral flow is immediately placed into the reader holder.

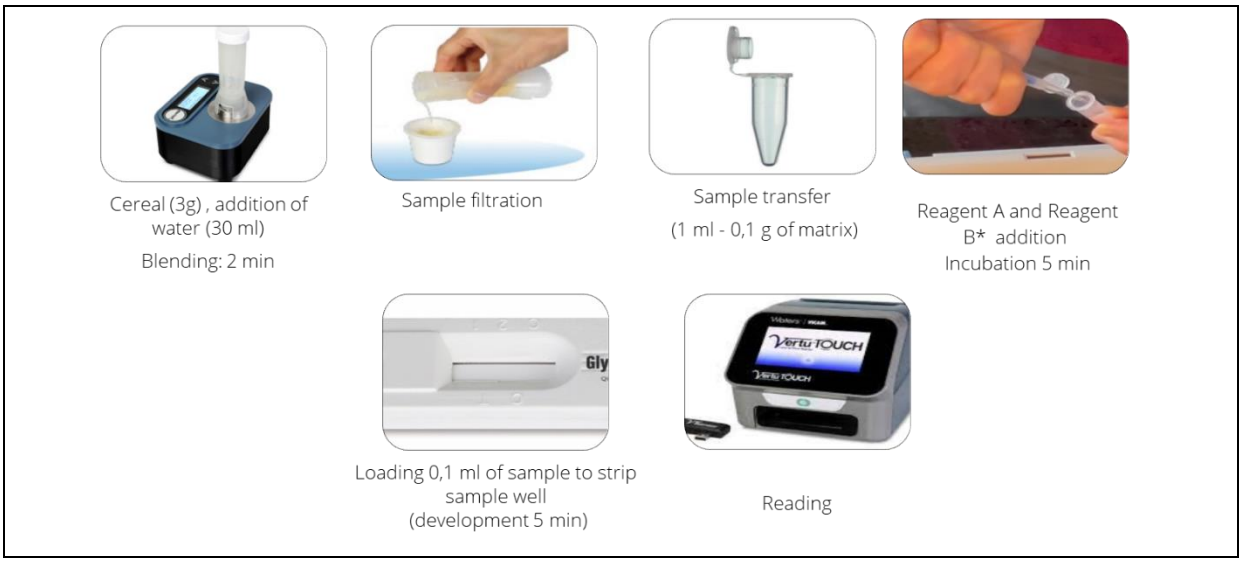

Figure 2: Optimized assay protocol

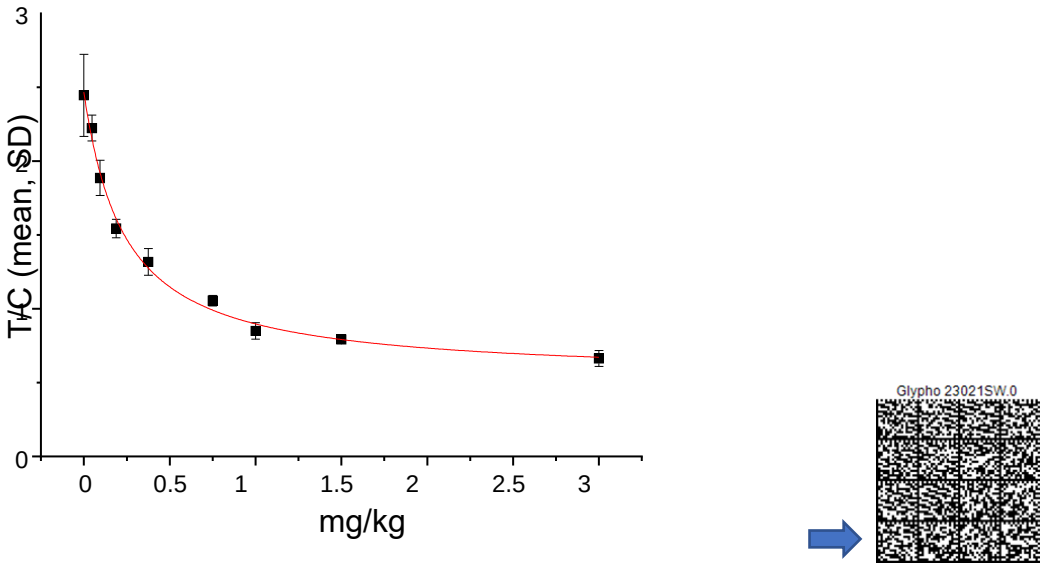

Figure 3: Calibration curve: T/T0 values on the y axis where T represents the intensity value of the T line and C represents the intensity value of the control line versus concentration on the x axis (ng loaded on test). Calibration curve specific for the commodity and a lot of strips is saved as QR code.

2.2 Testing of prototype stability

The evaluation of prototype stability consisted of testing of stability and storage condition of reagent solutions: reagent A (buffer solution) and reagent B[1] (derivatization solution). Tree different batches of reagent solutions were evaluated, specifically the storage condition and vial-opening stability (time that the reagents that cannot be used up in one time can be stored stably) were tested at room temperature and at refrigerate temperature (+4°). While reagent B did not present any stability problems, reagent A showed the formation of white precipitates when stored at 4 degrees, and once opened a short-term stability (1 month -Table 1). The white precipitate in reagent A could be attributed to a specific component of the reagent formulation affecting the fluidity of the migration from the sample pad. It was also observed that the presence of precipitate in reagent A affected the reproducibility of the test result.

		Room temperature	Refrigerate temperature (+4°C)
Opened vial (2 months)	**Reagent A**	✓	✗
	Reagent B	✓	✓
Closed vial	**Reagent A**	✗	✗
	Reagent B	✓	✓

Table1: Result of testing of reagent stability and storage condition (✓, stable, ✗ not stable)

[1] Reagent composition covered by confidentiality requirements

2.3 Evaluating assay sensibility and precision

Four different production lots of the prototype test (Lot A, B, C, D), resulting from different immunoreagents deposition on the strip membrane and different reagent batches, were tested for sensibility (IC 50) and precision (repeatability of the measurements).
To this purpose, a calibration curve was built plotting T/T0 values on the y-axis (where T represents the intensity value of the T line and C represents the intensity value of the control line) and the concentration on the x axis (ng loaded on test). As an example, the results obtained for the first tested lot (Lot A) was shown in Figure 4

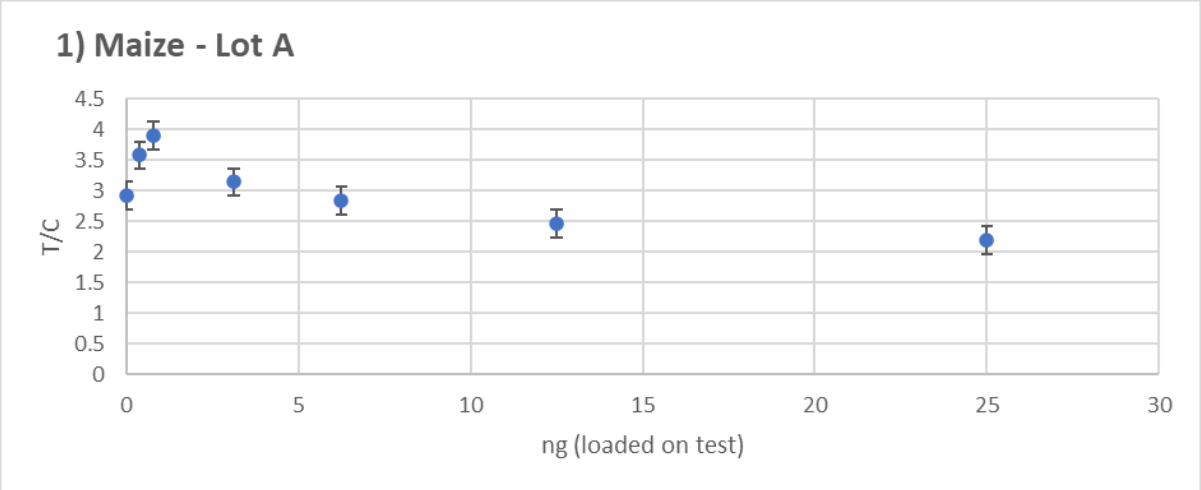

Figure 4: Calibration curve (T/C values on the y axis where T represents the intensity value of the T line and C represents the intensity value of the control line versus concentration on the x axis expressed in ng loaded on test) obtained with Lot A.

A preliminary estimation of the test sensitivity was evaluated spiking a maize sample at MRL and 0.5 MRL levels (Table 3). For each spiked extract, 5 independent lectures were carried out. The percentage of inhibitory concentration was evaluated according to the equation 1:

IC, % = (S0 –S)/S0 x 100

Where:

- S0: Ratio of test line (T) vs control line (C) of blank sample
- S: Ratio of test line (T) vs control line (C) in contaminated sample

The test sensibility was considered satisfactory if the half maximum inhibitory concentration (IC50, analyte concentration for which 50 % of the test signal is switched off) was achieved. The IC50 value is usually used as a preliminary estimation of the cut-off value for tests involving a visual reading of the strip. The CUT OFF level was the response, signal or concentration, obtained with the screening method, above which the sample is classified as “suspect”. In the case of tests involving an optical reader, such as the one used in this project, an IC30 value could also be used.

The lot A showed too weak inhibition of signal intensity in maize due to the matrix effect, such that only a % inhibition of 16 was achieved at MRL level (1mg/kg). The performances obtained were not considered satisfactory therefore new lots were tested (lot B, lot C, Lot D) characterized by a different immunochromatographic test.

Maize		Blank	0.5 MRL	MRL
		0	0.5 mg/kg	1 mg/kg
Lot A	T/C (Mean +SD)	2.93± 0.21	3.15± 0.23	2.49± 0.08
	% inhibition		-	16
Lot B	T/C (Mean +SD)	7.26± 1.19	4.69± 0.41	3.99± 0.38
	% inhibition		35	46
Lot C	T/C (Mean +SD)	2.91± 0.35	1.56± 0.17	1.16± 0.22
	% inhibition		46	60
Lot D	T/C (Mean +SD)	2.54± 0.08	1.42± 0.07	1.20± 0.09
	% inhibition		56	47

Table 2: T/C and % inhibition calculated on spiked extract maize (0.5 MRL and MRL) and blank sample

The obtained results were subjected to ANOVA TEST and Turkey test (P≤0.01) to evaluate if the tested lot could discriminate blank from 0.5 MRL and MRL. Lot B showed a significant difference between blank and 0.5 MRL and between blank and MRL. However, no significative difference between 0.5 MRL and MRL was found resulting in a risk of high false positive rate. The risk of high false positive rate may affect the test's economic benefit. Satisfactory precision was observed: precision (relative SD) ranged from 5% (for contaminated sample) to 19% (for blanks). Compared to previous lots, lot C showed a satisfactory % signal inhibition for maize at 0.5 MRL. The last lot tested (lot D) showed either a satisfactory percentage of inhibition and an improved precision respect all previously tested lots.

2.4 Building up matrix matched calibration

A preliminary analysis of spiked maize samples showed the need to use matrix matches calibration curves for accurate quantitative analysis.

Calibration curves in maize were prepared by serial dilution of a maize extract at 0.6 µg/mL (corresponding to 6.0 mg/kg) with blank maize in the following concentration range: 0.05, 0.09, 0.19, 0.38, 0.75, 1.50, 3.00 mg/kg. Each level was analyzed in triplicate by immunochromatographic test. The obtained calibration curve was uploaded into the reader as QR code.

To these purposes, standard solution of glyphosate (1000 mg/L. Water/Acetonitrile (9:1)), was purchased from Lab Instruments Srl (Castellana Grotte, Italy). Stock solutions of pesticide was prepared at concentration of 90 µg/mL.

The calibration curve obtained for Lot D in maize Below is reported below.

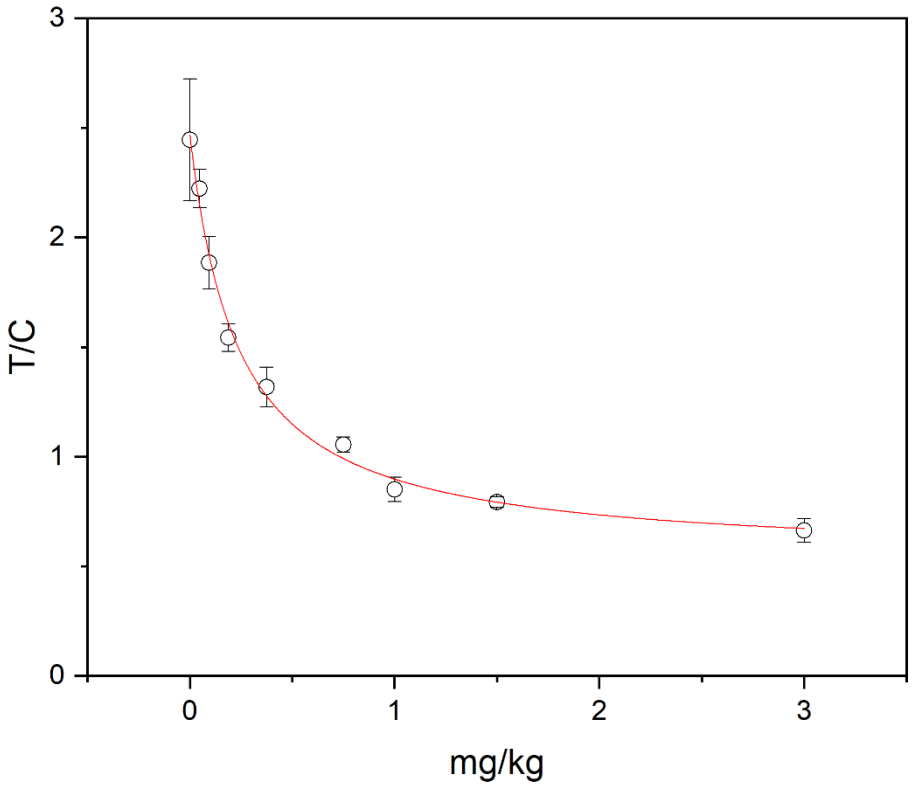

Figure 5: Calibration curve obtained for lot D (T/T0 values on the y axis where T represents the intensity value of the T line and C represents the intensity value of the control line versus concentration on the x axis (mg/kg) test).

An IC50 value equals to 0.23 ±0.04 mg/kg and an IC20 corresponding to 0.06 ± 0.01 mg/kg was estimated from the calibration curve. Lot D production parameters were fixed to produce a prototype suitable for validation experiments in maize, i.e. to discriminate samples contaminated at 100 %MRL (1 mg/kg), 50%, MRL (0.5 mg/kg) and 0% MRL.

In addition, preliminary tests were performed to verify if the optimized assay was able to discriminate maize samples contaminated at 100 %MRL (1 mg/kg), 10% MRL (0.1 mg/kg) – as candidate screening detection limit - and 0% MRL. Based on these preliminary results, the value of 0.1 mg/kg will be evaluated if suitable as a screening detection limit in the full validation experiments.

Maize		Blank	0.1 MRL	MRL
		0	0.1 mg/kg	1 mg/kg
Lot D	T/C (Mean +SD)	2.52± 0.38	1.83± 0.1	0.96± 0.10
	Concentration (mg/kg)		0.17± 0.05	1.3± 0.2

Table 3*: T*/C and concentration (mg/kg) calculated on spiked extract maize (0.1 MRL and MRL) and blank sample

2.4 Evaluating assay specifity

For cross reactivity studies, calibration curves (6 points) were constructed for each residue separately by serial dilution with milliQ water in the range 9.4 to 600 ng/mL (Figure 6). To this purpose single standard pesticide solutions of glyphosate, AMPA and N Acetyl glyphosate were prepared at 0.6 µg/mL by dilution of standard stock solution of glyphosate (1000 mg/L. Water/Acetonitrile (9:1)), AMPA (100 mg/L Water/Acetonitrile (9:1)) and N- Acetyl glyphosate (1mg). The pesticide standard solutions were purchased from Lab Instruments Srl (Castellana Grotte, Italy).
Cross reactivity was tested by comparing IC50 values obtained by calibration graphs built in milliQ water. The antibody showed cross reactivity of 92% for N-Acetyl glyphosate (IC50 is equals to 1.33 T/C for glyphosate and1.23 for N-acetyl glyphosat (see figure e, respectively) and no cross reactivity for AMPA. The absence of traces of glyphosate in the N-acetyl glyphosate was verified injecting standard solution of N-acetyl glyphosate at 0.6 ng/µl in LC-HRMS. (see section 2.5)

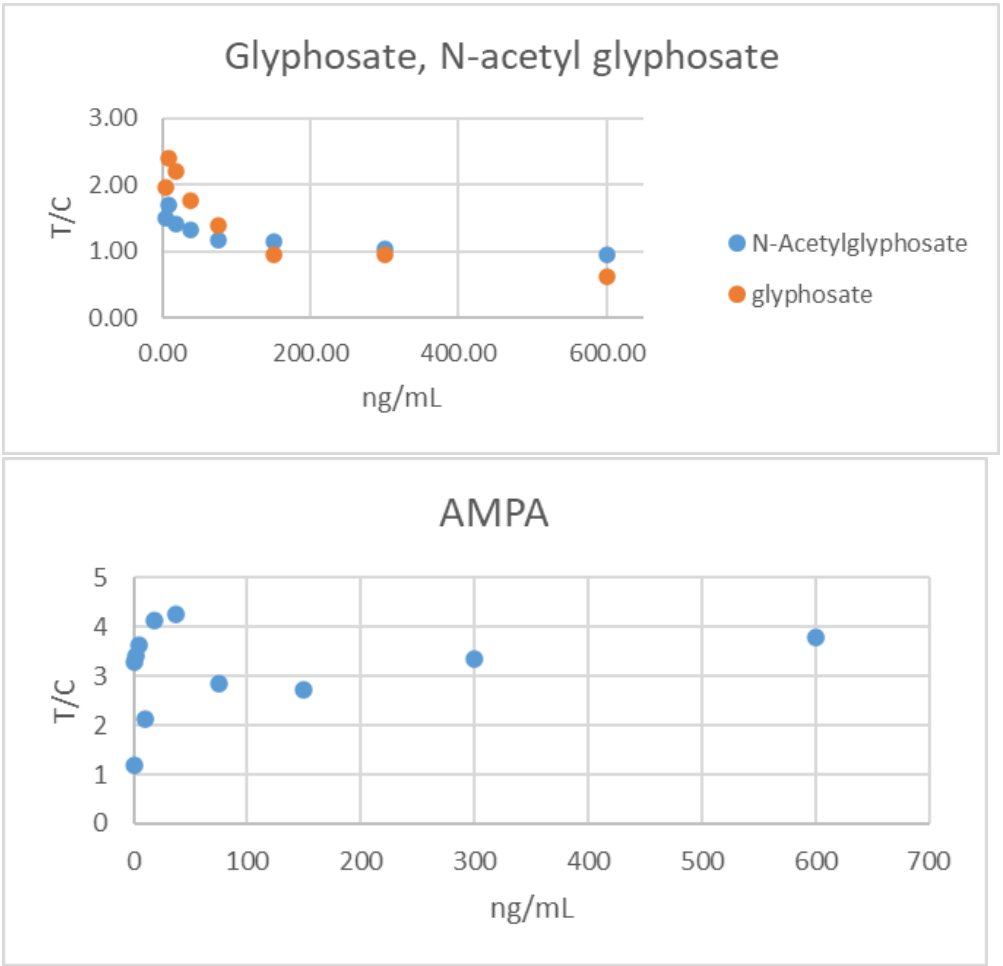

Figure 6: Standard calibration curves obtained for glyphosate, AMPA and N Acetyl glyphosate

2.5 LC-HRMS confirmatory analysis of glyphosate and metabolites AMPA, N-Acetyl AMPA

A confirmatory method was optimized and implemented to detect glyphosate and target metabolites in the samples to be used for the characterization of the validation sample set, trueness assessment, assessment of purity and stability of standard solutions.

Confirmatory analysis for glyphosate and metabolites were performed by LC-HRMS according to QuPPe method (for the simultaneous analysis of a number of highly polar pesticide) [6].
The detection of polar pesticides was achieved using ultrahigh-performance liquid chromatography coupled with quadrupole – orbitrap high resolution mass spectrometer (Orbitrap Exploris 240™, Thermo Scientific, San Jose, CA, USA). The instrument was equipped with a heated electrospray ionization (HESI) source. The ion transfer tube temperature and vaporizer temperature were set at 320 °C and 280 °C, while the electrospray voltage was set at 3.00 kV operating in negative mode. Sheath and auxiliary gas were set at 45 and 15 arbitrary units, with an S lens RF level of 70.A target SIM combined with a target MS2 for the confirmatory response, based on an inclusion list was used.The resolving power of FS was set at 60,000 Full Width at Half Maximum (FWHM), a scan range of m/z 50–250 was selected, the automatic gain control (AGC) was set as standard, and the maximum injection time was set in the auto mode. The MS2 operated at 15,000 FWHM. The AGC target was set in the standard mode, with an auto maximum injection time. The Q1 resolution was set at 1 m/z. Fragmentation of precursors was optimized as two-stepped normalized collision energy (NCE) (15 and 30 eV). The formula of the compound, with the exact theoretical mass of the parents and the diagnostic transition used to confirm Glyphosate, AMPA and N-acetyl glyphosate are reported in Table 3.

Compound	Precursor Ion			Product Ions (m/z)	
	Adduct	Formula	Exact mass	Exact mass	Formula
Glyphosate	$[M-H]^-$	$C_3H_8NO_5P$	168.0067	62.96417	$[O_2P]^-$
				124.01687	[C2H7O3NP]-
				149.99612	$[C_3H_7NO_3P]^-$
Glyphosate-2-^{13}C,^{15}N	$[M-H]^-$	$^{13}C_2C^{15}NH_8O_5P$	171.0105	62.96423	$[O_2P]$-
				80.97488	$[H_2PO_3]^-$
				153.00058	$[^{13}C_2C^{15}H_7NO_3P]^-$
AMPA	$[M-H]^-$	CH_6NO_3P	110.0013	62.96417	$[O_2P]^-$
				78.95904	$[O_3P]^-$
				80.97468	$[H_2PO_3]^-$
AMPA-$^{13}C^{15}N$	$[M-H]^-$	$^{13}CH_6{}^{15}NO_3P$	112.0016	62.96429	$[O_2P]$-
				80.97503	$[H_2PO_3]^-$
N-acetyl-glyphosate	$[M-H]^-$	$C_5H_{10}NO_6P$	210.0173	62.96421	$[O_2P]$-
				124.01717	$[C_2H_7O_3NP]^-$
				149.99636	$[C_4H_7O_3NP]^-$

Table 4. HRMS detection parameters.

The chromatographic separation was conducted using an Anionic Polar Pesticide (APP) column (5 µm, 2.1 mm × 150 mm) (Waters Corporation), which was thermostat at 50 °C. The chromatographic conditions were configured as follows: mobile phases included 0.9% formic acid in water (A) and acetonitrile acidified with 0.9% formic acid (B). The gradient reported in Table 1 was used as suggested in QuPPe method for APP column [6]. The entire run duration was 23 min, the flow rate was maintained at 0.500 mL/min, and 10 µL was the volume injected. The autosampler was held at 20 °C throughout the analysis.

	%A	%B
0	10	90
0.5	10	90
1.5	80	20
4.5	90	10
22.5	90	10
23	10	90
30	10	90

Table 5: LC Gradient as defined in the QuPPE Method

3 Conclusions

A prototype assay for glyphosate detection was developed and assessed according to the following steps:

- Optimization of sample preparation protocol for maize
- Testing prototype stability
- Building matrix matched calibration curves
- Evaluating sensibility and precision
- Evaluating assay specificity (cross-reactivity study)

Results of the evaluation of the above parameters showed the suitability of the developed assay for full validation in maize.
In addition, a LC-MS confirmatory method to be used in the validation study was implemented.

4. References

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