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Sequential determination of metabolites involved in the biosynthesis of aromatic amino acids after ultrasound-assisted extraction from plants and reverse LC separation

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ABSTRACT

A dual method is proposed for the determination of metabolites involved in the shikimate pathway which are biomarkers of the effects of glyphosate action on plants exposed to this herbicide. Extraction of the target metabolites (phenylalanine, tryptophan, tyrosine and shikimic acid) from a wheat model plant was accelerated by ultrasound energy. After centrifugation and micro-filtration, 1 μL of extract was injected into the chromatograph in an isocratic regime for 4 min to determine shikimate by absorption at 254 nm. In the mean time, a 130 μL aliquot of extract was subjected to derivatization with *o*-phthaldialdehyde and 2-mercaptoethanol for 1 min, the reaction stopped and 1 μL of the solution chromatographied in a gradient regime prior to laser-induced fluorescence detection of the derivatized amino acids. The characterization of the dual method provided limits of detection around 0.03 μg mL^{-1} for the aromatic amino acids and 1.52 μg mL^{-1} for shikimate, whereas the limits of quantitation ranged between 0.084 and 0.093 μg mL^{-1} for amino acids and was of 4.56 μg mL^{-1} for shikimate. The suitability of the method was checked by application to *Triticum aestivum* (wheat) plants grown under controlled conditions, sprayed with different doses of glyphosate and collected at different times after exposition to the herbicide.

1. Introduction

Glyphosate (*N*-phosphonomethyl glycine) is a broad-spectrum systemic herbicide used essentially on annual and perennial plants including grasses, sedges, broadleaf and woody plants. This non-selective herbicide is widely used on non-cropland and among a great variety of crops.

Glyphosate was first commercialized by Monsanto in 1974 under the name of Roundup® and thanks to its effectiveness and relatively low mammal toxicity became one of the most extensively used herbicides worldwide. The mechanism of glyphosate action is similar to that of other herbicides by suppressing the activity of a given enzyme. Particularly, glyphosate inhibits the action of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is involved in the shikimate pathway for synthesis of aromatic amino acids tryptophan, tyrosine and phenylalanine (Supplementary Fig. S1). The inhibition of EPSPS would involve accumulation of shikimate, the precursor of the pathway for synthesis of aromatic amino acids. In 1998, glyphosate resistance was first detected in several types of herbal plants [1], causing millonaire losses since this date.

Due to the wide use of glyphosate in agriculture, the interest in the development of analytical methods to detect and quantify its activity and that of its metabolites has grown. The methods proposed so far have been focused either on the glyphosate present in treated plants or on that which does not reach the target plant and accumulates in environmental media, especially water. In the former case, glyphosate must be removed from the vegetal tissues, task usually carried out by an extractant. Water is one of the most used extractants thanks to its green character and water solubility of glyphosate and its metabolites [2], but also organic solvents has been used with this aim [3].

Once glyphosate is in water, or when working directly with water samples, solid-phase extraction is the preferred technique for cleanup and preconcentration, thus obtaining the analytical sample [4] suitable for the selected detection technique [5–7].

Concerning the analysis step, among the numerous methods proposed so far, the reference method is that reported by Monsanto based on liquid chromatography (LC) separation, which involves a pre-column derivatization step, individual separation by LC and fluorometric detection of the resulting fluorofore [8]. Recently, a simple method based on capillary electrophoresis has

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been proposed for the simultaneous determination of glyphosate and all its metabolites (*viz.* aminomethylphosphonic acid – AMPA, glyoxylate, sarcosine and formaldehyde) in *Lolium spp.* plants [9]. Nevertheless, the most employed technique for determination of glyphosate and derivatives is LC coupled to mass spectrometry, since it provides good detection limits and has been successfully applied to different plant species such as tea [10] or soybeans [11]. LC has also been commonly used to determine glyphosate in waters [5,12,13] or in human fluids [14].

Another possibility to detect glyphosate application is to monitor the herbicide effects; thus is, the presence or evolution of biomarkers. Accumulation of shikimate by blockage of the aromatic amino acid biosynthesis is one of the most abundant methods in the literature for detection/quantitation of glyphosate [15–19].

Changes in free amino acids in plant extracts to predict the mode of action of herbicides was proposed in 1995, but, as far as the authors know, the use of an easy to apply and sensitive method for the determination of both biomarkers has not so far been reported [20]. Actually, most of the methods developed focus on either aromatic amino acids or shikimate.

Based on the above premises, the aim of the present research was the development of a method fast, simple and sensitive enough for the determination of both biomarkers by not costly equipment or reagents; then assessed by application to a number of samples.

2. Experimental

2.1. Reagents

Deionized water (18 MΩ cm) from a Milli-Q water purification system from Millipore (Molsheim, France) was used to prepare standards and solutions used in this research. D-phenylalanine (minimum purity 98%), D-tryptophan, D-tyrosine and shikimic acid (minimum purity 99%) were purchased from Sigma–Aldrich (Steinheim, Germany) to prepare solutions in deionized water. D-tyrosine stock solution was prepared in 0.2 M HCl aqueous solution because of the high solubility of this aromatic amino acid in acid medium. Stock solutions of all amino acids were prepared at 10 μg mL^{-1}, while working solutions were obtained by proper dilution of them.

o-Phthaldialdehyde (OPA) and 2-mercaptoethanol (ME) used as derivatization reagents were from Sigma–Aldrich (minimum purity 97%) and Merck (Darmstadt, Germany), respectively. The derivatization mixture was made by dissolving 0.135 g OPA in 5 mL methanol and made up to 25 mL with 0.1 M borate buffer adjusted to pH 9.0. Daily, 5 μL ME was added to 1 mL OPA solution and vortexed for 10 s. This mixture is the final derivatization solution.

Tetrahydrofurane (THF), sodium acetate and boric acid were from Panreac (Barcelona, Spain), while methanol was from Scharlab (Barcelona, Spain). All reagents were LC quality grade or higher.

Glyphosate (36% concentration) was obtained from Monsanto (St. Luis, MO, USA) as a commercial herbicide for plants treatment.

2.2. Samples

Seeds from *Triticum aestivum* L. (wheat) biotypes were collected for germination in Petri dishes using a damp filter paper as substrate. The seedlings were transplanted to plastic pots – three plants in each pot – filled with 1:2 (v/v) peat:clay as substrate. When plants averaged six leaves, commercial glyphosate was applied to 90% of the plants while the remainder 10% was used as control (blank). Application was performed by spraying at 200 kPa in a closed chamber calibrated at 0.5 m height above the target surface, with a relative volume of 200, 300 and 400 g a.i. Ha^{-1} (grams of active ingredient per hectare). Considering that the normal (commercial) application dose of glyphosate is 300 g a.i. Ha^{-1}, the concentrations were chosen to try the effects of sublethal and lethal doses on aromatic amino acids production. All wheat plants – control plants and those treated with herbicide – were harvested by cutting the aerial leaves after 4, 7 and 9 days since application, milled/crushed using a mortar and adding liquid nitrogen until the leaves presented a thin and homogeneous appearance, then stored at −40 °C until use. The 4 days time was selected to monitor the penetration of the herbicide in the plant at a sublethal dose (200 g a.i. Ha^{-1}) and at a dose over the commercial-recommended one (400 g a.i. Ha^{-1}), while longer times (7 and 9 days) allow to check the plant response to commercial doses (300 g a.i. Ha^{-1}) of glyphosate action.

2.3. Instruments and apparatus

A Branson Digital Sonifier (Danbury, CT, USA) equipped with an ultrasound probe connected to a conventional tip of 20 mm of diameter and an output power up to 400 W was used to assist extraction. The probe was hold by a wood- and cork-made box to avoid high operation noise.

An Agilent 1100 series (Palo Alto, *CA*, USA) high performance liquid chromatograph (LC) was used for chromatographic separation of the aromatic amino acids and shikimate precursor. The chromatograph was equipped with a module to operate at microscale regime with flow rates ranging from 1 to 20 μL min^{-1}. The injection was manually performed by a six-way/two-positions (load and injection) valve with automatic programmed switch. The sample loop had a volume of 1 μL, but smaller amounts of sample could be injected by turning back to the load position at preset times. A 10 μL syringe provided by SGE Analytical Science (Ringwood, Victoria, Australia) was used to fill the sample loop with the analytical sample. Silica-capillary tubes of 0.075 mm i.d. × 0.375 mm o.d. from Teknokroma (Sant Cugat del Vallés, Barcelona, Spain) were used to connect every module of the LC system, including detectors. To make possible the connection between the capillary and the different parts of the system, the ends of the former were protected by short tubes (3 cm length) of polyetheretherketone (PEEK) of 0.5 mm i.d., which were tightened using stainless-steel ferrules and connectors of 1/16" i.d. provided by Análisis Vínicos (Tomelloso, Ciudad Real, Spain). The chromatographic column was a Zorbax SB-C18, 5 μm 150 × 0.5 mm from Agilent.

The LC was equipped with a diode array detector (DAD) with a working wavelength range of 200–800 nm. The outlet of this detector was directly connected to a Zetalif 2000 325 nm/CE laser induced fluorescence (LIF) detector by the mentioned capillary, in which a detection window of 5 mm length was made for collecting the emitted light. Excitation of the derivatized metabolites was performed at 325 nm (nominal wavelength radiation provided by the laser source), and a photomultiplier detector collected the emission radiation without discrimination.

A centrifuge from JP Selecta (Barcelona, Spain) was used to separate solid fraction the extract. To avoid small size particles to flow into the μ-LC column, the extracts were then filtered using 0.22 μm pore size filters provided by Análisis Vínicos.

2.4. Proposed method

A volume of 0.2 mL, 10 mg L^{-1} taurine solution as internal standard (I.S.) was added to 0.25 g of milled plant sample into a test tube. Then, 5 mL 0.25 M HCl aqueous solution as extractant was added, vortexed and placed into a water bath, inside of which the ultrasound probe tip was plunged. The extraction was

developed for 10 min (at 70% of amplitude and 0.5 s s^{-1} of duty cycle). Then, the extract was separated from the solid by centrifugation at 1160 × *g* for 10 min. Prior to injection, the extracts required micro-filtration to avoid clogging of the LC system. First, 1 μL of extract was injected into the chromatograph to determine shikimate by absorption at 254 nm with the DAD. The chromatographic method used in this case was isocratic and the mobile aqueous phase consisted of 0.05 M sodium acetate (pH 6.8) plus 5% methanol circulated for 4 min. In the mean time, a 130 μL aliquot of extract was located in an 1.5 mL vial, 25 μL OPA–ME reagent mixture added and the resulting solution shaken. After exactly 1 min, the reaction was stopped by pH change adding 75 μL borate buffer prior to injection of the mixture (1 μL) for determination of the aromatic amino acids by monitoring the emission after laser excitation. A gradient program was used in this case consisting of an initial phase A (aqueous medium containing 0.05 M sodium acetate – pH 6.8 – and 5% methanol), and a phase B (methanol plus 0.5% THF), which changed as follows: from min 0 to 3 a 0% of mobile phase B; from min 3 to 30 phase B increased to 52%; and, finally, from min 30 to 42 an 80% of B was reached. The gradient steps were linear and the flow rate was 15 μL min^{-1}.

3. Results and discussion

3.1. Optimization of the derivatization step

Two different derivatization reagents and protocols were compared for analysis of aromatic amino acids using standard solutions. In the first, dansyl chloride (DNS) was used as reagent according to the following protocol [21]: 5 μL 0.5 M sodium bicarbonate buffer (pH 9.5) was added to 5 μL extract and finally, 5 μL of 1 mg L^{-1} DNS. Then, the mixture was held for 2 h at room temperature, after which, 0.1 mL ethyl acetate was added to extract the analytes.

The well known OPA–ME reagent mixture was also used for development of the protocol described in Experimental [22]. Preliminary studies showed that this derivatization protocol provided the best results in terms of signal, process time and simplicity, as the reaction takes 1 min *versus* the 2 h required for DNS derivatization.

The derivatization protocol is only applicable to the target amino acids since shikimate does not contain a chemical group able to produce a fluorescent product. For this reason, shikimate was determined by absorption taking advantage of its short chromatographic retention time (similar to the time required for derivatization of the target amino acids). Once the extract was obtained, shikimate was directly determined while aromatic amino acids were derivatized for their determination by LIF.

3.2. Optimization of the extraction step

All the experiments to optimize extraction were carried out using a control wheat plant (*i.e.* a non-treated plant) and aimed at the simultaneous extraction of all target analytes: shikimate and the aromatic amino acids. Influential extraction variables such as type of extractant and sonication conditions were evaluated to achieve the maximum extraction efficiency. The samples were spiked prior to extraction with a solution of taurine (5 μg mL^{-1}) as I.S. Four different extractants were tested (*viz.* 0.6% (w/v) NaCl, 0.6% (w/v) NaOH, 0.25 M HCl or 75% (v/v) ethanol) to check the influence of the extraction medium (*viz.* saline, alkaline, strong acidic and organic). Thus, four extraction tests under intermediate ultrasound conditions (50% amplitude, 0.5 s s^{-1} duty cycle of irradiation) and time (10 min) were carried out using each of the above solutions. The extraction was carried out in all cases with 0.25 g of plant material and 5 mL extractant. Fig. 1 shows the chromatograms obtained by injecting the four extraction tests, which revealed that a strong acid extractant favored isolation of shikimate and also that of aromatic amino acids, probably due to a better extraction of intracellular metabolites. In fact, this extractant has previously been employed for extraction of metabolites from plants [23].

Ultrasound parameters such as sonication amplitude and duty cycle were optimized by a bi-variant experimental design. Eleven experiments (including three central points) were performed within the amplitude range 30–70% and duty cycle range 0.2–0.8 s s^{-1} (second each second). The total extraction time

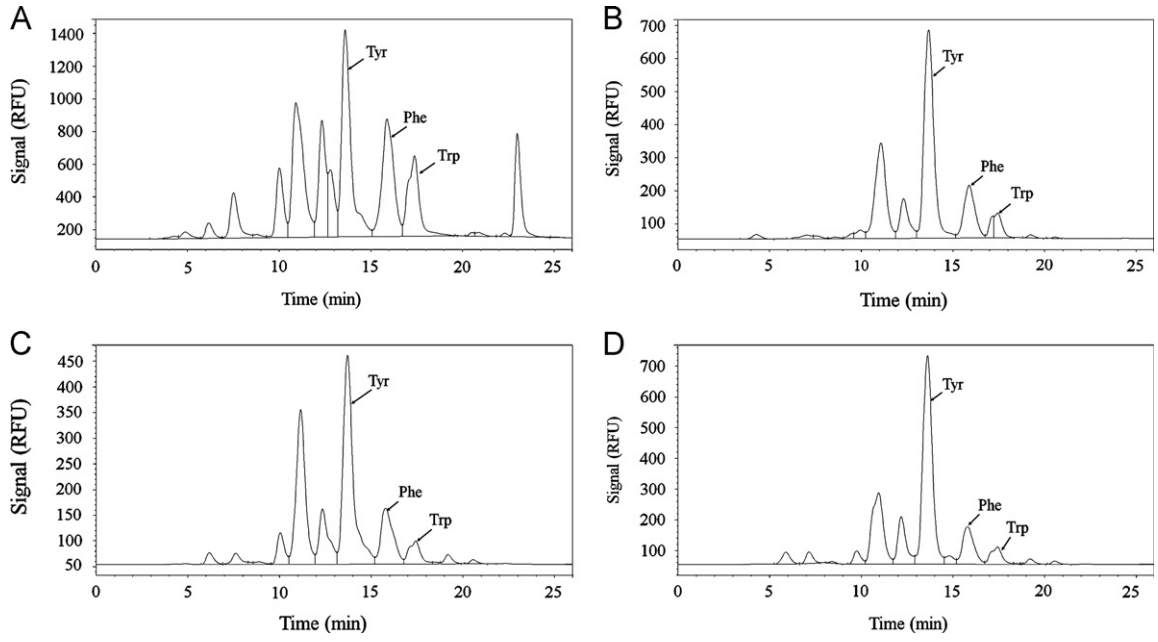

Fig. 1. Chromatograms from extracts obtained by the following extractants: (A) HCl, (B) ethanol, (C) NaCl, (D) NaOH. Tyr, Tyrosine; Phe, Phenylalanine; Trp, Tryptophan; RFU, Relative Fluorescence Units.

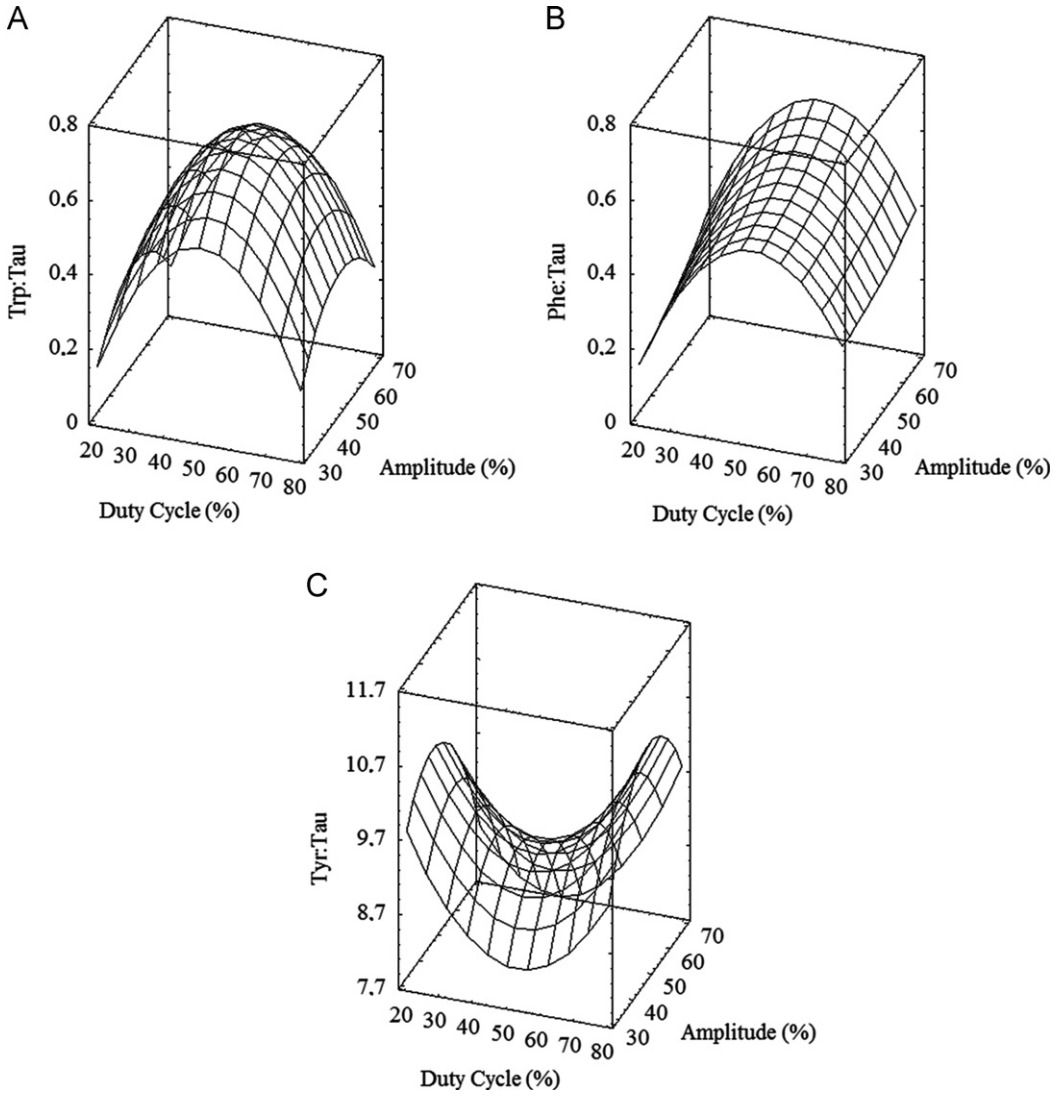

Fig. 2. Response surfaces for the optimization step of the ultrasound-assisted extraction for the three aromatic amino acids: (A) Tyr, (B) Phe, (C) Trp. Tau, Taurine (I.S.).

was fixed at 10 min. The response surface in Fig. 2 shows that the optimum extraction conditions for phenylalanine and tryptophan (Fig. 2A and B) were within the ranges studied. As can be seen, intermediate values of duty cycle (0.5 s s^{-1}) and high sonication amplitude (70%) led to the maximum extraction efficiency according to the response surfaces. For tyrosine, the optimal values are not clear to be within the ranges assayed (Fig. 2C) since two different trends can be observed. Anyway, this amino acid provides a high signal after derivatization and the best sensitivity (in terms of slope of calibration curve, see Table 1). This justifies why values of 70% amplitude and 0.5 s s^{-1} of duty cycle have been considered as the optimal ultrasonic conditions.

Optimization was completed with a kinetics study setting the optimum sonication parameters with the optimum extractant (0.25 M HCl) for 0, 2, 5, 10 and 20 min. Ten minutes was selected for further experiments as the extraction efficiency was significantly higher than that at 5 min (95% confidence level) only for tryptophan, and no statistical differences in extraction efficiency were found for longer extraction times.

The extraction was also carried out in the absence of ultrasound energy with 10 mL of 0.25 M HCl, 0.5 g of sample and shaking for 5 h (conventional method [24]). Comparison of the results showed that the proposed method requires a much shorter extraction time with a higher efficiency (10–25% higher, depending on the analyte).

3.3. Characterization of the dual method

The two parts of the determination method were characterized in terms of detection and quantification limits (LODs and LOQs), linear ranges, regression coefficients, precision and recovery for each of the target analytes.

The LODs and LOQs were expressed as the mass of analyte which gives a signal that is 3σ and 10σ, respectively, above the mean blank signal (where σ is the standard deviation of the blank signal). Thus, LODs were around 0.03 μg mL^{-1} for the aromatic amino acids and 1.52 μg mL^{-1} for shikimate, whereas LOQs ranged between 0.084 and 0.093 μg mL^{-1} for amino acids and 4.56 μg mL^{-1} for shikimate. Considering the low injection volume that the μLC device allows working with (1 μL), the on-column LOQs of the metabolites quantified in this study were lower than 1 ng for aromatic amino acids and around 4.5 ng for shikimate. The regression coefficient (R^2) was above 0.99 for shikimate and the three aromatic amino acids, as shows Table 1.

A precision study was carried out by analysis of five replicates of the same plant for three consecutive days. The results,

Table 1
Analytical characteristics of the DAD method (for shikimate determination) and LIF method (for aromatic amino acids).

Analyte	Equation	R^2	Linear range[a]	LOD[a]	LOQ[a]
Shk	$y=4.641x-23.223$	0.9984	5–100	1.52	4.56
Tyr	$y=0.329x-0.068$	0.9914	0.1–7	0.028	0.084
Phe	$y=0.3x-0.06$	0.9922	0.1–7	0.031	0.093
Trp	$y=0.228x-0.06$	0.9938	0.1–7	0.03	0.09

[a] Concentration expressed as μg mL^{-1}.

Table 2
Repeatability and reproducibility of the methods, expressed as percent of relative standard deviation (RSD).

Precision	Tyr	Phe	Trp	Shk
Day 1	7.57	15.13	12.00	4.03
Day 2	4.70	13.36	11.16	3.06
Day 3	6.06	7.95	6.05	4.15
Interday	8.29	16.40	12.01	4.10

Table 3
Extraction recovery (%) for isolation of the target metabolites at two concentration levels expressed as percentage.

Spiked level, %	Compound			
	Tyr	Trp	Phe	Shk
50	105.98	87.38	98.04	104.06
100	101.00	84.34	96.00	98.69

expressed as percent of relative standard deviation (% RSD), ranged between 3.1% and 15.1% for repeatability and between 4.11% and 16.4% for reproducibility, as can be seen in Table 2.

The study of the recovery was performed by analysis of cultivated wheat samples spiked at two levels (50% and 100% of the original concentration), and the recoveries ranged between 84% and 106%, as listed in Table 3. The recovery factor is an indicator of the accuracy of the method and the high values obtained allow confirming that there is not degradation of the metabolites during the ultrasound-assisted extraction process, at least within the values of time, duty cycle and amplitude tested.

3.4. Application of the dual method to the determination of the target metabolites in plants treated and non-treated with glyphosate

For checking the suitability of the dual method, it was applied to study the effect of glyphosate on the synthesis of aromatic amino acids and their precursor, shikimate, in the biochemical pathway. With this purpose, plants of wheat treated with glyphosate at a sublethal and two lethal doses and non-treated plants were grown as explained in Samples. Non-treated plants were collected for each treatment to compare the results obtained as a consequence of the herbicide application. The application study encompasses:

1. Comparison of the effect of a sublethal dose (application of 200 g a.i. Ha^{-1}) with a lethal dose of 400 g a.i. Ha^{-1} 4 days after glyphosate application and the non-treated plant. Table 4A shows the results for the four metabolites (using I.S. for the aromatic amino acids), while Supplementary Table S1 summarizes the percentage of aromatic amino acids and shikimate remaining in the plants after the three treatments with glyphosate taking as 100% that existing in the non-treated plants. As can be seen, a significant decrease in the concentration of aromatic amino acids was observed with the lethal dose as compared to the sublethal dose. Under the former, the concentration of tyrosine and tryptophan was reduced up to a 35% of the concentration present in the non-treated plant. The decrease was less relevant for the sublethal dose, in which both amino acids were decreased up to 71% and 79% of the reference concentrations. Concerning phenylalanine, it is interesting to point out the lowest influence of the glyphosate action on this amino acid with a decrease of only up to 90% and 60% of the concentration present in non-treated plants.
2. Study of the dose commercially recommended with lethal purposes for application in crops (300 g a.i. Ha^{-1}) at long times (7 and 9 days) after application as compared with non-treated plants. This study was aimed at measuring the target metabolites 9 days after application of this dose. After 7 days treatment, the concentration of amino acids decreased significantly up to 54%, 60% and 84% for tyrosine, phenylalanine and tryptophan, respectively. The analysis of the plants 9 days after glyphosate application revealed a persistent effect on the target amino acids, as can be seen in Table 4B and Supplementary Table S1.

Table 4
Concentration (μg mL^{-1}) of aromatic amino acids and shikimate in treated and non-treated wheat plants.

(A) Short-term effect with sublethal and lethal doses				
Time-dosis from sample treatment	**Tyr**	**Phe**	**Trp**	**Shk**
Non-treated – 4 days	5.89	1.02	1.27	490.07
Sublethal dose 200 g a.i. Ha^{-1} – 4 days	4.22	0.92	1.01	524.43
Lethal dose 400 g a.i. ha^{-1} – 4 days	2.02	0.64	0.44	535.26
(B) Long-term effect with commercial dose				
Non-treated – 4 days	5.89	1.02	1.27	490.07
Sublethal dose 200 g a.i. Ha^{-1} – 4 days	4.22	0.92	1.01	524.43
Lethal dose 400 g a.i. ha^{-1} – 4 days	2.02	0.64	0.44	535.26

Regarding shikimate, its concentration increased in a range between 7% and 26%, where the highest concentration corresponded to wheat plants collected 9 days after glyphosate application. The lowest concentration was found in plants treated with the sublethal dose and sampled after 4 days, as foreseeable.

These results can be visualized in the chromatograms shown in Figs. 3 and 4, thus demonstrating that the joint determination by the proposed dual method for shikimate and aromatic amino acids in plants allows monitoring the exposure grade to glyphosate.

4. Conclusions

A reliable approach is proposed here for the sequential determination of shikimate and essential amino acids after ultrasound-assisted extraction of the target metabolites in plants. First, the determination of shikimate is carried out in a time short enough (4 min) to be developed during amino acids derivatization. The characteristics of the method allow its application for monitoring the effect of glyphosate on the shikimate pathway at both sublethal and lethal applications.

The pathway of aromatic amino acids formation is proportionally inhibited as much as glyphosate is applied to the plants, resulting in a decreased concentration of amino acids formation and an increase in the concentration of their precursor.

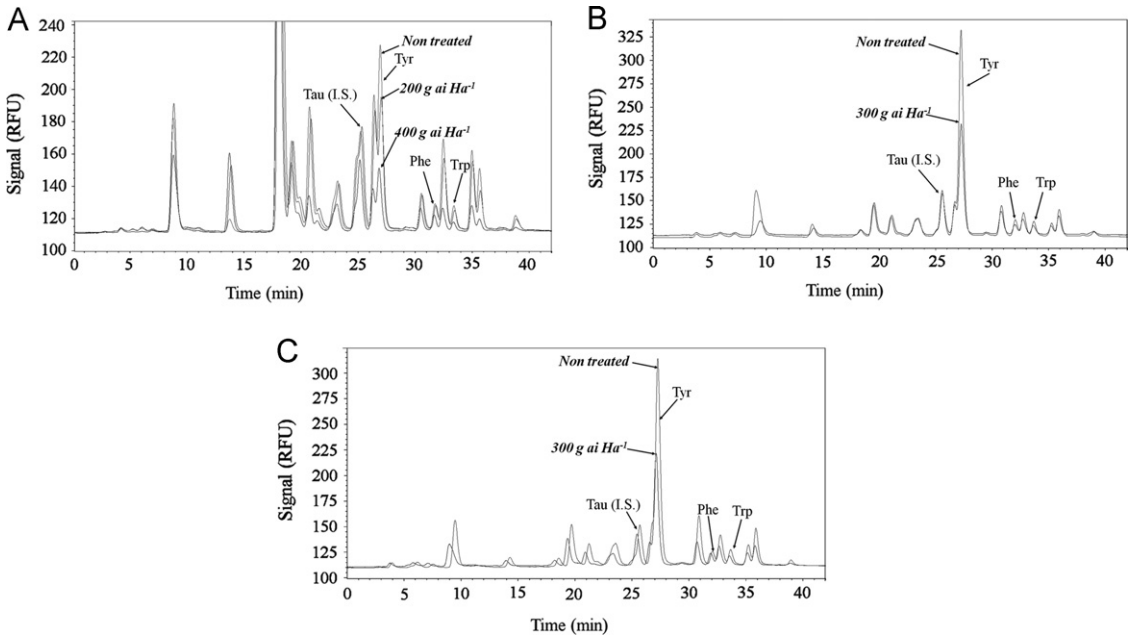

Fig. 3. Overlaid LIF chromatograms (amino acids) obtained from wheat samples under different conditions. (A) Non-treated, and treated with doses 200 g a.i. Ha^{-1} and 400 g a.i. Ha^{-1} after 4 days application. (B) Non-treated, and treated with doses 300 g a.i. Ha^{-1} after 7days application. (C) Non-treated, and treated with dose 300 g a.i. Ha^{-1} after 9 days application.

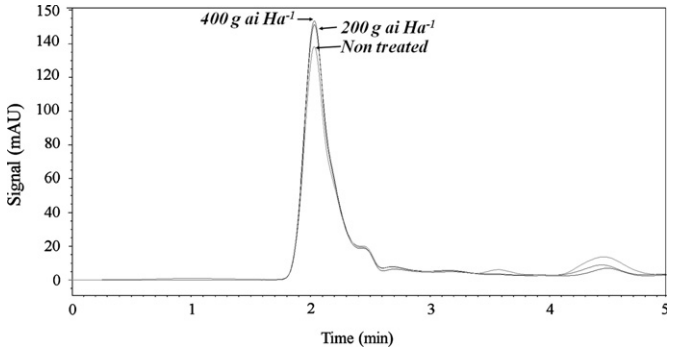

Fig. 4. Overlaid DAD chromatograms of shikimate (Shk) obtained from samples under different conditions. Non-treated, and treated with doses 200 g a.i. Ha^{-1} and 400 g a.i. Ha^{-1} after 4 days application. mAU: Mili absorbance units.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.10.077.

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