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Screening and confirmatory analysis of glyoxylate: A biomarker of plants resistance against herbicides

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ABSTRACT

The evidence that glyoxylate is a biomarker of tolerance or susceptibility to the action of herbicides belonging to the glycine family makes necessary to develop simple methods for the determination of this metabolite. Glyoxylate level allows both to know the presence/absence of members of the glycine family in plants and plant response to these herbicides. With this aim, a colorimetric-screening method has been developed for determination of glyoxylate based on formation of a phenylhydrazone, then oxidised to red coloured 1,5-diphenylformazan. Simultaneous optimization of ultrasound-assisted extraction of glyoxylate from plants and derivatization by a multivariate design has allowed the determination of the target analyte in fresh plants without interferences from pheophytines and compounds with carbonyl groups. Limits of detection and quantification are 0.05 $\mu g\,ml^{-1}$ and 0.17 $\mu g\,ml^{-1}$, respectively, with precision, expressed as relative standard deviation, of 3.3% for repeatability and 5.6% for the within-day laboratory reproducibility. Only 50 mg of plant is necessary for determination of glyoxylate within 32 min. Confirmatory analysis by capillary electrophoresis-diode array detection in samples of *Lolium spp.* subjected to treatment with glyphosate shows that the relative error of the proposed method is always lower than 7%.

1. Introduction

Glyoxylate, a metabolite endowed with aldehyde and carboxylate groups, is involved in different metabolic pathways in bacteria, plants and animals [1–5]. There are two main sources of glyoxylate formation in plants. One comes from photorespiration, through the activity of glycolate oxidase; the other from the glyoxylate cycle that bypasses the steps in the citric acid cycle where carbon is lost as CO_2 [6]. Several enzymes (mainly dehydrogenases and transaminases) are involved in the synthesis and catabolism of glyoxylate [7–12]. These biocatalysts can be inhibited, or even mutated, by the action of external agents such as herbicides or by internal agents such as feedback, in both cases with lethal effects on the regulation of glyoxylate levels [13–15]. For this reason, glyoxylate could be considered a biomarker to detect these abnormal enzyme behaviours and, therefore, the development of methods for screening and confirmatory analysis of this metabolite are of interest.

Glyoxylate has been determined in different matrices, from biological fluids such as urine or blood [16–19] to plant extracts [20,22]. Among the different methods, one alternative is that reported by Kramer et al. [21] for glyoxylate determination in aqueous solutions. This method is based on the formation of glyoxylate phenylhydrazone and subsequent oxidation, in acid medium, to 1,5-diphenylformazan, an intensely coloured red compound with maximum absorption at 520 nm. The main limitation of this approach is the lack of selectivity since any molecule with carbonyl groups could interfere the determination of glyoxylate. The method was improved by Bräutigam et al. for simultaneous determination of glyoxylate and ammonium in tobacco plants by inclusion of liquid–liquid extraction with chloroform [22]. The toxic character of chloroform as extractant together with a long extraction time and number of steps are the main drawbacks of this method. Glyoxylate has also been determined using separation techniques prior to detection such as GC [16], LC [17,18] or CE [20,23]. These methods require more sophisticated, no portable instrumentation and, usually, they do not provide a fast response; aspects which are critical for in-field methods.

An approach is here proposed for screening and confirmatory analysis of glyoxylate in extracts from different parts of the leaf treated with glyphosate, a representative of the glycine herbicide family. The screening method is a modification of the colorimetric test reported by Kramer et al. [21]. The main variables influencing the derivatization reaction, as well as those involved in the extraction of the target analyte from plants, have been optimized using multivariate approaches to minimize or avoid the influ-

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ence of interferences for more accurate and simple determination of glyoxylate and for quantitative extraction from the samples, respectively. The approach has been completed with a confirmatory method based on capillary-electrophoresis separation of glyoxylate from plant extracts and diode-array detection/quantification.

2. Materials and methods

2.1. Reagents

Potassium ferricyanide, methanol and hydrochloric acid from Panreac (Barcelona, Spain) and glyoxylate sodium salt and phenylhydrazine from Sigma (St. Louis, USA) were used in the screening method. Stock-standard solutions were prepared by dissolving 0.1 g glyoxylate in 100 ml deionized water (18 mΩ cm) from a Millipore Milli-Q water purification system.

Acetone, sodium hydroxide and acetonitrile from Panreac, and hexadecyltrimethylammonium bromide (CTAB) from Fluka (Buchs, Switzerland) were also used. Glyphosate (36% concentration) was obtained from Monsanto (St. Louis, USA) as a commercial herbicide for plants treatment.

2.2. Samples

Seeds from different *Lolium spp.* biotypes were collected in different geographical zones of Spain 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. 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 l ha^{-1}. All plants – treated with herbicide and control – were cut after 0, 24, 48, 72 and 96 h since application and stored at −40 °C until use. The sample thus prepared was used for screening analysis. For confirmatory analysis, the plant was placed in a porcelain mortar, flash-frozen using 20-ml liquid nitrogen, grinded to a fine powder using the porcelain pestle for 5 min and stored at −40 °C until use.

2.3. Instruments and apparatus

Herbicide was applied to plants located in a closed spray chamber furnished with a flat jet-spray tip Teejet 80.02 (Wheaton, USA). Eppendorf vials (Eppendorf GA, Germany) were used in the extraction and derivatization steps. A thermostated centrifuge Beckman Coulter Avanti J-25 (Fullerton, USA) furnished with a rotor no. 20 and an ultrasonic bath from Selecta (Barcelona, Spain) – generator power 50 W –, equipped with a temperature control unit were used in the extraction step. Nylon filters 45-μm pore-size and 13 mm i.d. from Millipore (Carrigtwohill, Ireland) were used to remove solid particles. A thermostated water-bath from Selecta (Barcelona, Spain) was used for acceleration of extraction and derivatization steps.

A 3D Capillary Electrophoresis Agilent G1600A Instrument, equipped with a diode array detector (DAD, range 190–600 nm); capillary tubing of 88.5 cm (effective length 80 cm) × 50 μm i.d. × 375 μm o.d. (Tecnokroma, Spain), and thermostated by a Peltier unit, was used for confirmatory analysis of glyoxylate. The instrumental setup was controlled by the Agilent ChemStation software, which also enabled data acquisition and processing. A 8453 Agilent diode-array spectrophotometer (190–1100 nm) was used to obtain absorption spectra.

2.4. Screening method

50 mg of the target plant and 1-ml water are put into contact in an Eppendorf vial, which is subject to ultrasound for 10 min at 20 °C. Two 450-μl aliquots of the extract are used: each aliquot is mixed with 50 μl of a freshly prepared 1% (w/v) solution of phenylhydrazine in 100 mM HCl, and kept at 60 °C for 10 min; then acidified by adding 250-μl concentrated HCl. 100 μl of an 1.6% (w/v) $K_3Fe(CN)_6$ in water solution is added to one of the aliquots (aliquot A), and 100 μl distilled water to aliquot B and the absorbance of both aliquots is monitored at 520 nm after reaction development for 12 min. The difference in absorbance between aliquots A and B corresponds to glyoxylate extracted from the given plant. The scheme of the overall process is in Fig. 1.

2.5. Confirmatory method

The method used for confirmation of screening is that proposed by the authors for determination of glyphosate, glyoxylate, sarcosine, aminomethylphosphonic acid and formaldehyde, based on ultrasound-assisted extraction and separation-quantification by capillary electrophoresis-diode array detection [23]. In this method, 1.5-g sample is transferred to a plastic beaker and extracted three times with 8-ml 1:1 water–acetone, each time involving magnetic stirring for 10 min, ultrasonication for 5 min, and centrifugation at 4 °C and 12,096 × *g* for 15 min. The supernatants obtained with the three 8-ml portions extractant are pooled and evaporated to dryness under nitrogen flow. The extract is reconstituted with 2-ml background electrolyte (BGE) (10 mM potassium phthalate, 0.5 mM CTAB and 10% acetonitrile at pH 7.5) and filtered through a nylon filter before CE analysis. The filtered extract is injected for 5 s at −10 kV into the BGE. The analysis voltage is −20 kV, and the monitoring wavelength selected for indirect detection of all analytes 220 nm. To maintain the capillary under optimal working conditions, its surface was regenerated after each run by sequential washing with water (2 min), 0.1 M sodium hydroxide (2 min), 1 min waiting, and BGE (10 min). In addition, the capillary was activated everyday by sequential washing with water (1 min), 0.1 M sodium hydroxide (10 min), 5 min waiting, and water (1 min).

3. Results and discussion

3.1. Optimization of the method for screening of glyoxylate

The method reported by Kramer et al. for the determination of glyoxylate was applied only to analysis of water [21]. Use of this method after extraction of the target analyte from plants requires the joint optimization of the extraction and derivatization steps to assure the highest efficiency of glyoxylate isolation with minimum interferences from species with carbonyl groups, because of the scant selectivity of the derivatization reaction. Extraction of glyoxylate from plants is influenced by temperature, process time, pH of the extraction medium (HCl concentration), and applied auxiliary energy. Meanwhile, the derivatization step is influenced by incubation temperature and reaction time, in addition to reagents concentration.

Preliminary experiments on extraction in the presence and absence of ultrasound energy showed that this energy accelerates sample leaching, which is complete after 10 min, causes degradation for longer irradiation times, and modifies the presence of interferents in the extract. The time required for a similar extraction efficiency using magnetic stirring is 40 min.

After checking both that the concentrations of reagents proposed by Kramer et al. are in a high excess with respect

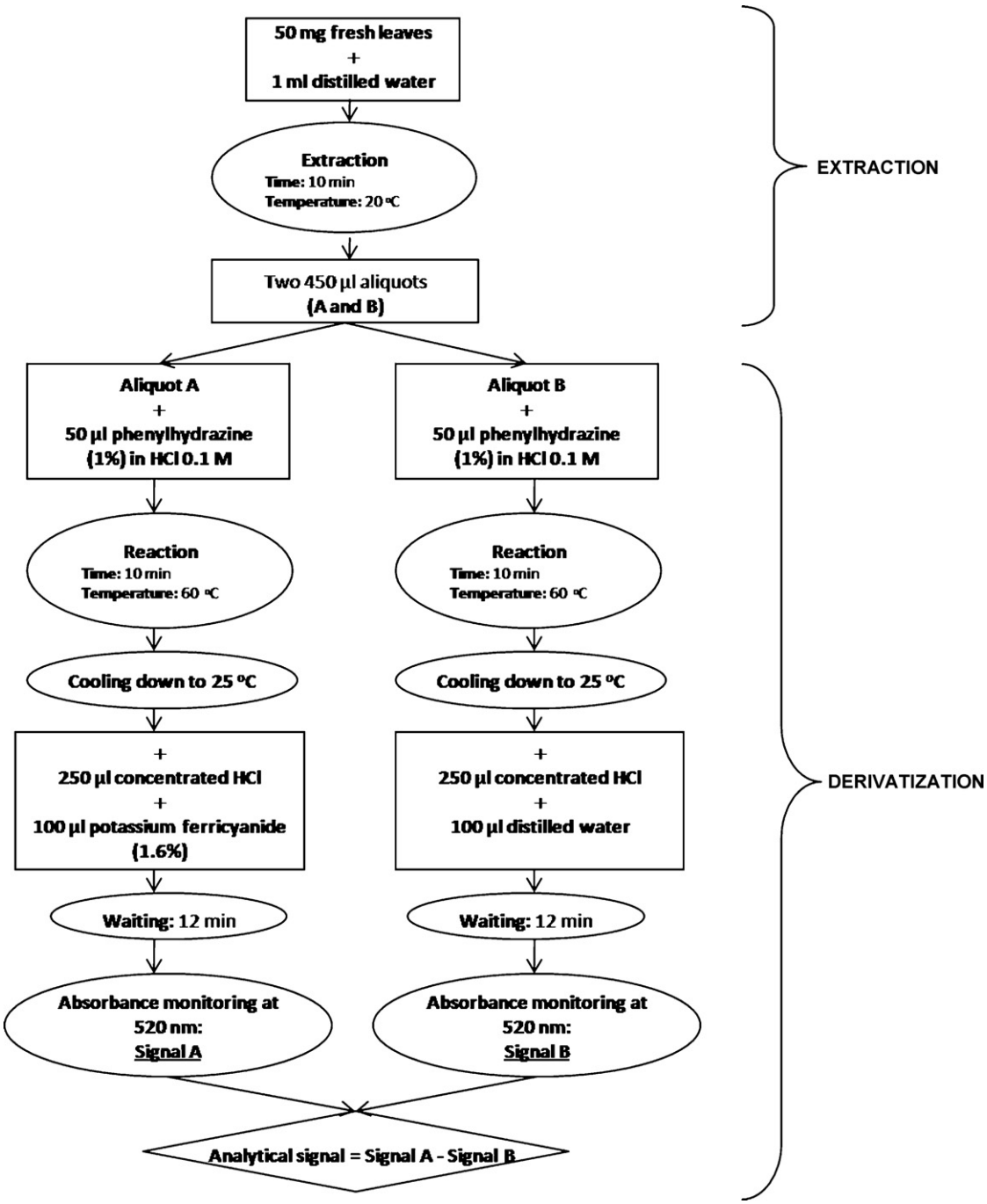

Fig. 1. Scheme of the proposed screening method.

to that of glyoxylate in the extract from plants and none of the reagents absorb at the monitoring wavelength, these concentrations were adopted for the screening method. Thus, the optimization study was centered on extraction temperature, extraction time, hydrochloric acid concentration in the extractant, temperature of the first step of the derivatization reaction development (to avoid reaction of other species with carbonyl groups present in the extract), and derivatization reaction time (because of the instability of the reaction product). These influential variables were optimized by a multivariate approach using as response variable the difference in absorbance between the two aliquots (A and B) used for each optimization point: one in which the 1,5-difenilformazan is formed after derivatization and the other, corresponding to the background signal (contribution from other species absorbing at 520 nm), without addition of the redox solution which gives place to the coloured compound from the target analyte. Therefore, the 1,5-difenilformazan formation could be maximized (both by favouring analyte extraction and formation of the coloured reaction product); while the background signal (contributing pheophytins formed in acid medium by conversion of extracted chlorophylls) could be minimized.

The use of a multivariate experimental design enabled drastic reduction of the number of experiments to be carried out. First, a factorial design involving 19 experiments and 3 central points provided information about the most influential variables as well as their possible interactions, so that the main effects and interactions were statistically evaluated. Fig. 2 shows the variables found to be significant for extraction and derivatization of glyoxy-

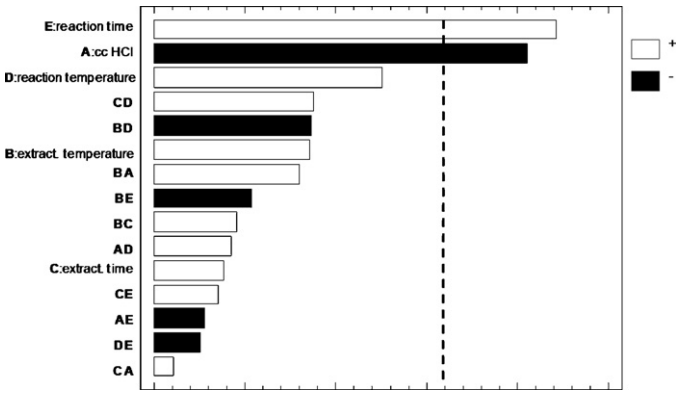

Fig. 2. Significance level of each variable studied in the screening experimental design for extraction and derivatization variables. Only two variables are significant: reaction time (positive effect) and HCl concentration (negative effect).

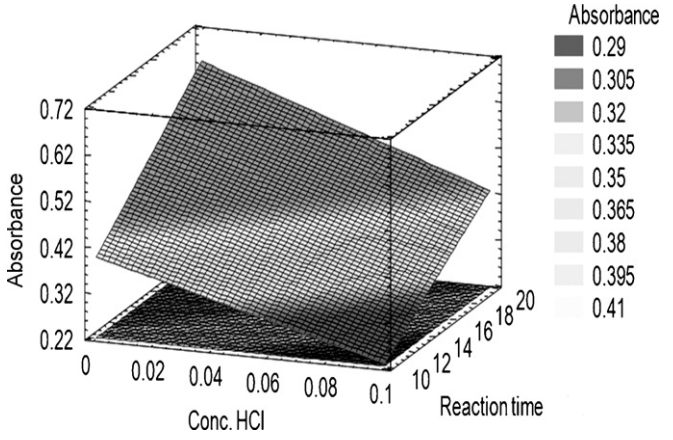

Fig. 3. Contours of the estimated response surface in the second experimental design. The optimum values (maximum absorbance) are represented by the less coloured area of the figure, indicating the values of the variables to which they must work to obtain the maximum signal.

late, which were the concentration of HCl in the extractant and the time for development of the first step of the derivatization reaction; that is, after addition of phenylhydrazine. The acid concentration showed a negative effect, while the effect of the reaction time was positive. Therefore, the variable response increased by lowering acid concentration and increasing the reaction time. Non-significant factors were fixed at their optimum values, which were extraction temperature 20 °C, extraction time 10 min, and temperature for formation of the phenylhydrazone (after addition of the phenylhydrazine solution): 60 °C. The two significant variables were subsequently studied by a surface-response design in which they were in the range 0–0.1 M for hydrochloric acid and 10–20 min for reaction time. The estimated response surface in Fig. 3 shows a better behaviour without acid addition and 20 min incubation time. The optimum development of extraction in the absence of HCl in the extract is in contradiction with usual favourable effect of acids in extraction media for isolation of intracellular metabolites. This behaviour can be explained because chlorophylls have scant solubility in water and, in addition, their undesirable conversion to pheophytins (with absorption at the monitoring wavelength of the target reaction product) is favoured in an acid medium. Therefore, the interference of pheophytins is avoided at a close-to-neutral pH and ambient temperature in order to avoid chlorophylls extraction. In this way, the signal from aliquot B is drastically decreased and, thus, the difference between signals from aliquots A and B increased.

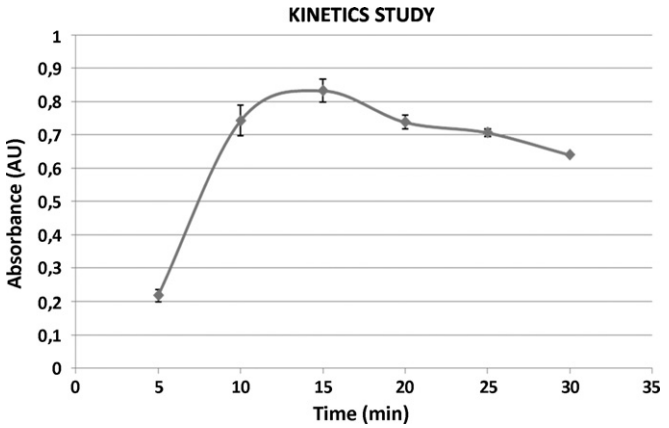

Fig. 4. . Kinetics study of the redox reaction. Degradation of the formazan complex starts 12 min after mixing with the oxidant (potassium ferricyanide).

Optimization was completed with a kinetics study of the last step of the derivatization reaction because the monitored product showed instability. As Fig. 4 shows, 12 min, from mixture of the reactants to monitoring, is the time necessary to obtain an almost plateau resulting from the equilibrium between formation of the reaction product and its degradation. After this period, degradation predominates over formation and the absorbance of the analytical signal decreases.

3.2. Validation of the proposed approach

Calibration plots were run for glyoxylate using the absorbance data as a function of the standard concentration of glyoxylate. The calibration equation, regression coefficient and linear dynamic range are listed in Table 1. The limit of detection (LOD), expressed as the concentration of analyte providing a signal 3σ above the mean blank signal, where σ is the standard deviation of the blank signal, was 0.05 μg ml^{-1}. The blank signal was obtained from plants which were not treated with glyphosate. The limit of quantification, LOQ, expressed as the concentration of analyte which gives a signal 10σ the mean blank signal, was 0.17 μg ml^{-1} (see Table 1).

Within laboratory reproducibility and repeatability were evaluated for the proposed method in a single experimental setup with triplicates by experiments carried out with natural samples—a pool of plants from four weed biotypes of *Lolium spp.* cut 96 h after glyphosate application. Three measurements of these samples per day were carried out on 3 days. Eq. (1) was used to determine the between-day variance:

$$s^2_{\text{between}} = \frac{\text{MS}_{\text{between}} - \text{MS}_{\text{within}}}{n_j} \quad (1)$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and n_j is the number of replicates per day. The within-laboratory reproducibility, s^2_{WR}, was calculated by Eq. (2).

$$s^2_{\text{WR}} = s^2_{\text{r}} + s^2_{\text{between}} \quad (2)$$

where s^2_{r} is the residual mean squares within-days and s^2_{between} is the variance due to the between-day effect.

The results obtained are listed in Table 1. The repeatability, expressed as relative standard deviation (RSD), is 3.3% for glyoxylate in this pool of plants; the within-day laboratory reproducibility, also expressed as RSD, is 5.6%.

3.3. Study of potential interferences in the analysis of glyoxylate

After eliminating interferences such as those caused by chlorophylls and their degradation products by optimization of the

Table 1
Features of the two methods for a pool of natural samples cut 96 h after glyphosate application.

Method	Calibration equation[a]	R^2	Linear range	LOD[b]	LOQ[b]	Average[c]	S_b[d]	S_{wr}[d]
Screening	$Y = 0.0678 + 0.0886X$	0.997	LOQ-600	0.05	0.5	38.2	3.3	5.6
CE	$Y = 0.641 + 0.244X$	0.998	LOQ-500	0.2	0.7	2.7	2.6	5.0

[a] Y expressed as absorbance units $\times 10^{-3}$; X as $\mu g\,ml^{-1}$.
[b] Expressed as $\mu g\,ml^{-1}$.
[c] Expressed as $\mu g\,g^{-1}$.
[d] Expressed as %.

extraction step, other potential interferences of the derivatization reaction were studied.

The derivatization reaction between phenylhydrazine and glyoxylate and subsequent oxidation with formation of the coloured compound is common to other compounds with carbonyl groups such as aldehydes and ketones, but in this case, with yellow colour. Therefore, compounds usually present in plants extracts such as sugars (glucose, fructose) or derivatives (ascorbic acid) could be potential interferents in the determination of glyoxylate. An interference study was carried out spiking glucose and fructose at concentrations ranging from 0.1 to 1000 mg ml^{-1} to a 5-mg ml^{-1} glyoxylate standard solution. The average measurement resulted in a glyoxylate concentration of 5.026 ± 0.139 mg ml^{-1}, which involved a relative standard deviation below 3%. This enabled to conclude that there is no interference at the monitoring wavelength.

3.4. Influence of sample humidity

The humidity content of the samples is other relevant factor which deserved to be studied because of a drying step (to homogenize humidity between different plants). Heating could favour the conversion of chlorophylls into pheophytins, the latter being soluble in the extractant medium. The influence of sample humidity was tested by experiments involving portions of *Lolium spp.*, which were cut and independently dried at temperatures of 30, 60 and 70 °C for 12 h. Fig. 5 illustrates the absorbance at 520 nm of aliquots A and B of extracts (that is, with and without reaction with ferricyanide). As can be seen, the absorbance in aliquots B (caused by the presence of pheophytins in the extract) is drastically influenced by the drying temperature that influences the formation of pheophytins, which is nil in fresh material. This is an additional key favourable aspect of the method, which allows the plant to be subject to extraction without any prior treatment.

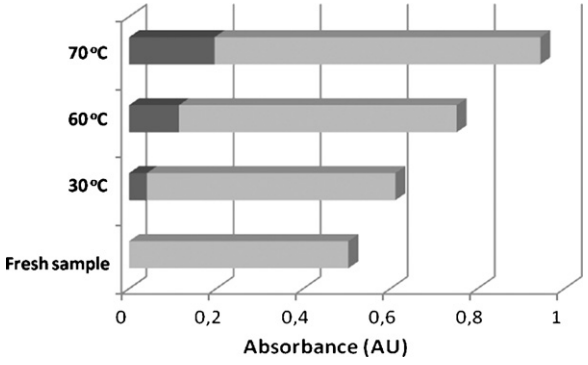

Fig. 5. Changes in absorbance at 520 nm of the extracts from fresh samples and samples dried at 30, 60 and 70 °C prior to (dark zone) and after (clear zone) reaction with potassium ferricyanide. The extracts from dried samples are coloured due to the presence of pheophytins. The absorbance of these compounds is summed up to that from the product of the derivatization reaction.

3.5. Sampling study for glyoxylate determination

To ensure the homogeneity of the samples when taken from different parts of the leaf, a study of glyoxylate distribution on the leaf was performed. Fig. 6A shows the different sampling areas of leaves selected for this study: cotyledon, apex, midrib and stem; while Fig. 6B shows the glyoxylate concentration as a function of the sampled area. As can be seen, the concentration of glyoxylate was significantly higher in the apex area, behaviour ascribed to the fact that the apex is the part of the plant more exposed to light, with a higher photorespiration activity.

3.6. Confirmatory analysis

The confirmatory analysis of the proposed screening approach is based on CE, which allows determination of glyoxylate in plants together with glyphosate aminomethylphosphonic acid, sarcosine and formaldehyde, previously proposed by the authors [23]. Both screening and confirmatory methods were applied to the same samples following the procedures under "Experimental". Table 2 shows the results provided by both methods, while Table 3 summarizes the differences between the two methods based on the equation of relative error (in which the data provided by the CE method are considered as the true value of the concentration of glyoxylate in the samples).

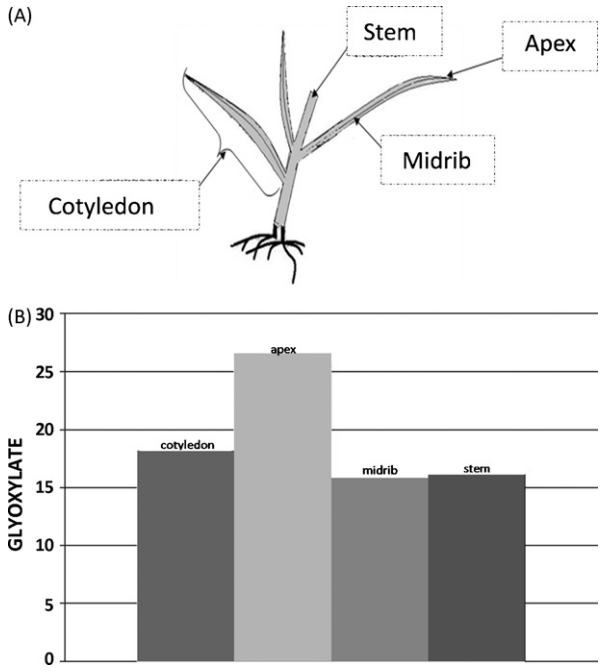

Fig. 6. (A) Scheme of *Lolium spp.* signaling the different parts selected for determination of glyoxylate. (B) Accumulation of glyoxylate in the different parts of *Lolium spp.* As can be seen, the accumulation of glyoxylate is higher in the apex, while it is lower in midrib and stem, both with similar accumulation.

Table 2
Analysis by the screening and CE methods of glyoxylate in samples cut 0, 24, 48, 72 and 96 h after glyphosate application ($n = 9$ replicates).

Samples	Concentration of glyoxylate[a]					Method
	0 h	24 h	48 h	72 h	96 h	
Lolium 1	13.794	27.256	34.479	55.909	51.545	Screening
	14.738	29.236	34.173	56.752	52.432	CE
Lolium 2	25.962	59.453	42.169	53.411	58.761	Screening
	26.745	60.2949	43.053	54.063	59.648	CE
Lolium 3	51.944	23.561	38.189	46.165	27.256	Screening
	52.727	24.506	39.172	47.029	28.148	CE
Lolium 4	27.211	94.096	105.059	130.77	103.922	Screening
	28.096	94.981	105.904	132.046	104.931	CE

[a] Expressed as $\mu g\,g^{-1}$.

Table 3
Difference between the screening and the CE methods based on the equation of relative error (data from the CE method are considered as true value).

Sample	Relative error (%)				
	0 h	24 h	48 h	72 h	96 h
Lolium 1	6.399	6.772	0.896	1.485	1.691
Lolium 2	2.929	1.396	2.0529	1.207	1.488
Lolium 3	1.486	3.855	2.510	1.837	3.171
Lolium 4	3.152	0.932	0.798	0.966	0.961

4. Conclusions

The screening method here proposed allows a fast determination of glyoxylate in plants treated with herbicides using simple instrumentation. In addition to its simplicity and rapidity, the method is endowed with appropriate reproducibility and sensitivity, so it can be used to monitor changes in the level of glyoxylate (or other members of the glycine family) in plants treated with herbicides, thus relating variations in the level of this metabolite with the application of herbicides.

The in-depth optimization study has allowed elimination of interferences and avoidance of pretreatment steps, thus making possible the development of the method in 32 min, which in turn makes possible its simultaneous application to a high number of samples in a short time, as desirable for a screening method.

Application of this method to obtain information of the mode of action of herbicides in different plants (both resistant and susceptible to herbicides) is our present research, to demonstrate the usefulness of the method in agronomic studies.

Conflict of interest

The authors declare no conflict of interest.

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