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New Phytochemical Screening Method for Biomarkers in Plants Exposed to Herbicides

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Traditionally, screening methods have been used to study pharmacological effects of phytochemical compounds. Nomura et al. investigated the chemistry of phenolic compounds of licorice (*Glycyrrhiza* species) and screened for their estrogenic and cytotoxic activities (Nomura et al. 2002). Koleva et al. used different methods to screen for antioxidant activity of extracts from *Sideritis* species (Labiatae) (Koleva et al. 2002 and 2003) and Couladis et al. screened Greek aromatic plants for antioxidant activity (Couladis et al. 2003). However, screening methods such as advanced chemical screening using High Performance Liquid Chromatography (HPLC) together with a mass spectrometer (MS) assays have also been used to detect phytochemical compounds as phenylpropanoids in cereals (Bily et al. 2004). Raharjo and Verpoorte presented four different methods as e.g. gas chromatography (GC), for analysis of Cannabinoids in biological materials (Raharjo and Verpoorte 2004).

Several definitions of a biomarker have been suggested in the literature (Hugget et al. 1992; Ernst and Peterson 1994; Ernst 1999; Walker 1995; Goldstein et al. 1987). However, in this paper, a biomarker pattern is defined as the changes in the composition and content of phytochemical compounds detected in plants after exposure to herbicides.

In this study, we present a novel and simple phytochemical screening method using High Performance Planar Chromatography (HPPC) to detect biomarkers in raw ethanol extracts of plants exposed to a herbicide and to identify biomarkers in different phytochemical groups by their functional groups.

MATERIALS AND METHODS

In this study, we tested one terrestrial plant species exposed to glyphosate in 40 different HPPC-systems with 2 stationary phases, 2 mobile phases and 10 different derivatization reagents in different combinations. The HPPC-Systems were classified according to the group of phytochemical compounds detected. The derivatisation reagent A: Carbohydrates: Thymol-sulphuric acid (Adachi 1965); B: Carbohydrates, N-containing compounds and terpenoids: Anisaldehyde-sulphuric

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acid (Lisboa 1963, modified); C: Organic acids, lipids and terpenoids: Molybdatophosphoric acid (Kritschevsky and Kirk 1952); D: Terpenoids: Zinc chloride (Stevens 1964 modified); E: All compounds with natural colour in both visual and UV-light: no derivatisation; F: Alcohols and phenolic compounds and terpenoids: 2-aminoethyl diphenylborinat (Neu 1957, modified); G: S-containing compounds: Methylene blue (Crépy et al. 1964, modified); H: Organic acids, lipids and terpenoids: Rhodamine 6G (Witter et al. 1957, modified); I: N-containing compounds, organic acids, lipids, terpenoids: Bismuth(III)nitrate-potassium iodide (Munier and Macheboeuf 1951); J: Organic acids and lipids: Bromocresol green-bromophenol blue-potassium permanganate (Pásková 1960); K: Amino acids: Ninhydrin (copper(II)nitrate)(Kawerau and Wieland 1951, modified). This was done in order to test the variability of a large number of different biomarkers.

Plants of *Apera spica-venti* (L.) were grown in 2L pots in a potting mixture consisting of soil, sand and peat (2:1:1 w/w %) containing all necessary macro- and micro-nutrients. The pots were placed in a heated glasshouse (14°C) with supplemental artificial light (16 hours photoperiod). The pots were sub-irrigated twice a day with de-ionised water.

Herbicide application was carried out at the four-leaf stage. A dose of 360 g ha^{-1} of glyphosate (Roundup Bio, 360 g a.i. L^{-1}, Monsanto Crop Sciences Denmark A/S) was applied in deionized water using a laboratory pot sprayer fitted with two Hardi-ISO F-110-02 flat fan nozzles in a spray volume of 145 L ha^{-1}. The plants were harvested 14 days after exposure and immediately freeze-dried and kept dry protected to light before phytochemical screening.

All chemicals and solvents used were of analytical grade and were obtained from Merck, VWR-International (Albertslund, Denmark). Standards and reagents were obtained from SIGMA-ALDRICH (Copenhagen, Denmark) and ACROS, Bie & Berntsen A/S (Rødovre, Denmark).

Three groups of replicates of control- and exposed plant were prepared using crushed freeze-dried plant material. Fifty mg plant material per ml 75% ethanol was extracted for 2 hours in an ice-cooled ultrasonic bath (Bransonic®, Danbury, USA). The extracts were centrifuged in a Micro Centrifuge (Capsule, Japan) at 6400 rpm for 10 minutes and immediately used for analysis. Extracts of the plants were placed on the Thin Layer Chromatography (TLC) plates 1.5 cm from the bottom of the plate using disposable Blaubrand® (IntraEnd, Wertheim, Germany) micropipettes in amounts of 5µl (plate A) and 10µl (plate B), both with ± 0.5% accuracy. The TLC plates were eluted in CAMAG flat bottom TLC chambers (Muttenz, Switzerland) containing 50ml solvent. One-dimensional TLC was performed until the solvent front was 0.5 cm from the top of the TLC-plate. The TLC-plates were then air-dried for one hour before derivatization.

Several High Performance Planar Chromatography (HPPC) systems were used.

Each HPPC-system is composed of a TLC plate (20 cm x 10 cm), a chromatographic solvent, and a derivatization reagent. Two combinations of TLC plates and solvents were used while the derivatization reagents varied between the forty test systems. The stationary phases were plate A made of silica gel 60 on High Performance Thin Layer Chromatography (HPTLC) aluminum sheets (No. 1.05547 Merck) and plate B made of cellulose on TLC aluminum sheets (No. 1.05552 Merck). The mobile phases were: Solvent 1 consisted of 1-butanol:acetic acid:water in a ratio of 4:1:5 (upper phase after shaking for 5 minutes) and 2 consisted of 1-propanol:25% ammonia/water in the ratio 11:9. All the derivatization reagents (except for A and B at plate type B) were used in each HPPC-System, and hereby 40 different combinations of the screening method were used. The combination of the stationary- and mobile phases with different pH-values was optimized to separate phytochemical compounds present in the plant extracts. However, the screening method was not developed for quantification, since the separation was not optimal for all compounds in the plants. Different derivatization reagents were used to detect different functional groups of biomarkers. Rf-values (Gänshirt, 1969) described the location of the biomarkers on the HPPC-plates.

For the detection of biomarkers, the plates were photographed using CAMAG VideoStore 2 Version 3.00 software using CAMAG Ultra Violet (UV) Lamp (254 nm, 366 nm and white light), a CAMAG Reprostar 3 with a HV-C20A Hitachi camera fitted (Canon TV zoom lens 11.5-69 mm). CAMAG VideoScan Programme Version 1.01 software was used for computer analysis. R_f-values were calculated as $R_f = D_1 D_2^{-1}$, where D_1 is the spot centre from the start point and D_2 is the distance of the solvent front from the start point (Gänshirt 1969). The visual intensity (VI) of the biomarkers were evaluated as n.d. = not detected, 1 = slightly visual, 2 = visual, 3 = medium visual and 4 = strongly visual. After analysis the TLC plates were fixed on paper, coated with Ibico laminating pouches using Ibico IL-12Hr laminator, speed 2m sec^{-1} at 110°.

The identification of biomarkers was performed using standards. Rf-values, color reaction in different HPPC-systems and UV-spectra of both standards and the biomarkers from CAMAG TLC Scanner 3 were used for identification.

With respect to the interpretation of the results, the same biomarkers can be detected in several of the HPPC-Systems. The same R_f-value will then be registered. In this case the different chemical reagents in the HPPC systems will detect the biomarkers, which have different chemical reactive groups. However, it may also be that two different biomarkers are detected at the same R_f-value. Since the HPPC screening system methods were not optimal for all the compounds detected in the different plant species, the compounds may be hidden behind each other and will therefore not be detectable with all the reagents used in this study. For the HPPC-system with the derivatization reagent C (ninhydrin), free amino acids are primarily detected. Similarity studies, comparing the R_f-values and the color of pure standards of amino acids with the detected biomarkers indicate

which amino acid is detected as a biomarker.

RESULTS AND DISCUSSION

The phytochemical screening method was developed to provide a new simple method to detect phytochemical compounds, present in high concentration in ethanol/water extracts of herbicide exposed plants. More than 25 different combinations of stationary- and mobile phases were tested to achieve the best separation of detectable phytochemical compounds and to determine the differences between the control plants and the herbicide treated plants. To find the best solvents the combination of the polarity of the solvents was used according to Snyder (Snyder 1974). Using acidic and basic solvents increased the number of compounds detected in one screening procedure. Compounds are often stable in acidic solvents, but may compose in basic solvents. The ionization of the functional groups during development of the plates in two different types of solvent did also have an important perspective related to the position of the compound (Rf-values) on the plates. The screening method was developed using extracts of 10 different plant species representing both aquatic- and terrestrial plant species and mono- and dicotyledons, exposed to different kinds of herbicides. In this paper, however, only the results of the grass *Apera spica-venti* (Poaceae) are presented. To investigate the best extraction solvent, water, ethanol/water in different concentration and pentane were tested. The extracts using 75% etahnol/water represented the best view of content of phytochemical compounds, since polar- and partly apolar compounds were extracted in this solvent. The content of e.g. flavanoids and other phenolic compounds was much higher for extraction with 75% ethanol compared with water and pentane. However, for extraction of carbohydrates and amino acids, water or 10% ethanol was the best extraction solvent. The stationary phases with silica gel 60 and cellulose in combination with the mobile phases of 1-butanol: acetic acid: water (4:1:5) and 1-propanol: 25% ammonia (11:9) gave the best separation of the detectable phytochemical compounds after derivatization with the different reagents. The screening method was developed to detect different functional groups of the phytochemical compounds using the same stationary- and mobile phases for each derivatization reagents. However, chemical reagents containing sulphuric acid could not be used with the stationary phase of cellulose, since the cellulose carbonized. The chemical reaction of the phytochemical compounds with the derivatization reagents facilitates the identification of the chemical structure of the biomarkers. More than 26 different modified chemical reagents were investigated to detect a broad spectrum of phytochemical compounds in the extracts. All the compounds with natural UV-fluorescence or absorption were detected without any use of reagents and they were detected in both visual light and UV-light at 254 and at 366 nm. Chlorophylls were detected as green spots in visual light and red spots in UV light, and carotenoids were detected as yellow to orange spots in visual light (Stahl 1969). The amino acids were detected as red, yellow and violet spots on a white plate in visual light using ninhydrin stabilized with copper (Kawerau and Wieland 1951). Shikimic acid was detected in visual

light as a remarkable pink spot on silica gel 60 developed in butanol, acetic acid and water with the chemical reaction with anisaldehyde after heating. The spot turned green during cooling.

All the biomarkers detected and identified in extracts of *A. spica-venti* exposed to glyphosate are presented in table 1 to 4.

Table 1. Biomarkers detected in all the thin layer chromatography systems with the stationary phase: HPTLC aluminium sheets silica gel 60 Merck 1.05547 and the mobile phase: 1-propanol: 25% ammonia (11:9) and all derivatization reagents.

Control VI	Exposed VI	Rf-value	Suggested Compound	Derivatisation Reagents Used for Detection
n.d.	4	0.02 +/- 0.01	-	A(vis); D(254); B(vis)*
2	n.d.	0.20 +/-0.01	-	B(vis); J(vis)*
n.d.	2	0.22 +/- 0.01	-	J(vis)*
n.d.	4	0.37 +/- 0.02	-	A(vis); D(254)*; C(vis)*; B(vis)
n.d.	4	0.51 +/- 0.02	Shikimic acid	B(vis)*; J(vis)
n.d.	4	0.52 +/- 0.01	-	B(vis)*; E(366)*; A(vis); K(vis)
n.d.	4	0.52 +/- 0.01	Glutamine	K(vis)*
n.d.	4	0.54 +/- 0.01	Proline	K(vis)*
n.d.	4	0.58 +/- 0.01	-	F(366)*; H(366); G(366)
4	n.d.	0.62 +/- 0.01	-	F(366)*; H(366); G(366)
4	n.d.	0.65 +/- 0.02	-	G(366); A(vis); C(vis)*; B(vis)*; F(366)*; E(366); I(366); H(366)
1	4	0.69+/- 0.02	Valine	K(vis)*
4	n.d.	0.70 +/- 0.02		G(366); J(vis)*; C(vis)*; B(vis); D(366); E(366); A(vis); H(366)
n.d.	4	0.75 +/- 0.01		F(366)*; D(254)*; G(366); E(366); I(366)
n.d.	4	0.75 +/- 0.01	Leucine/ Isoleucine	K(vis)*
n.d.	4	0.76 +/- 0.02	-	D(254)*; E(366)*; G(366); F(366)
4	n.d.	0.86 +/- 0.01	-	D(254)*; B(vis); C(vis)*; A(vis)
4	1	0.95 +/- 0.02	Chlorophyll	B(vis); F(366)*; D(254); A(vis); J(vis); E(366); K(vis); C(vis); H(366); G(366)

Table 2. Biomarkers detected in all the thin layer chromatography systems with the stationary phase: TLC aluminium sheets cellulose plate Merck 1.05552 and the mobile phase: 1-propanol: 25% ammonia (11:9) and all the derivatization reagents except A and B.

Control VI	Exposed VI	Rf-value	Suggested Compound	Derivatisation Reagents Used for Detection
4	n.d	0.02 +/- 0.01	-	D(254); F(366)*; C(vis); E(366)*
4	n.d	0.07 +/- 0.01	-	D(254); F(366)*; E(366); H(366); G(366)
4	n.d	0.11 +/- 0.01	-	D(254); F(366)*;G(366); E(366); H(366); D(366)
4	n.d	0.20 +/- 0.01	-	D(254); F(366)*; G(366);E(366); H(366); D(366)
n.d	4	0.22 +/- 0.01	-	F(366)*;G(366); E(366)
4	n.d	0.38 +/- 0.03	-	F(366)*;I(366); E(366)*;H(366)
n.d	4	0.41 +/- 0.01	-	C(vis)*
n.d	4	0.41 +/- 0.01	Glutamine	K(vis)*
n.d	4	0.45 +/- 0.02	-	D(254); F(366)*; I(366); E(366); J(vis); H(366)
n.d	4	0.54 +/- 0.01	-	D(254); F(366)*; E(366)*; H(366); G(366)
1	4	0.54 +/- 0.01	Threonine	K(vis)*
3	n.d	0.57 +/- 0.01	-	F(366)*; I(366); G(366)
n.d	4	0.61 +/- 0.01	-	C(vis)
n.d	4	0.61 +/- 0.01	Proline	K(vis)*
3	n.d	0.62+/- 0.01	-	F(366)*; E(366)*
n.d	3	0.78 +/- 0.02	-	G(366)
1	3	0.78 +/- 0.02	Leucine/ Isoleucine	K(vis)*
3	n.d	0.84 +/- 0.01	-	I(366)
4	1	0.95 +/- 0.02	Chlorophyll	D(254); F(366)*; K(vis); I(366); C(vis); E(366)*; H(366); J(vis); G(366)

Table 3. Biomarkers detected in all the thin layer chromatography systems with the stationary phase: HPTLC aluminium sheets silica gel 60 Merck 1.05547 and the mobile phase: 1-butanol:acetic acid:water (4:1:5) (upper phase) and all the derivatization reagents.

Control VI	Exposed VI	Rf-value	Suggested Compound	Derivatisation Reagents Used for Detection
1	3	0.02 +/- 0.00	-	A(vis); B(vis)*; C(vis)
n.d.	4	0.07 +/- 0.01	-	D(254); E(366)*
n.d.	4	0.07 +/- 0.01	Glutamine	K(vis)*
n.d.	4	0.10 +/- 0.02	-	B(vis)*; A(vis)*; J(vis)*; C(vis); D(254)
1	4	0.10 +/- 0.02	Proline	K(vis)*

n.d.	4	0.18 +/- 0.01	-	I(254); D(254); E(254)*; F(366)*; G(366); H(366)
1	n.d.	0.25 +/- 0.01	-	C(vis)
n.d.	4	0.32 +/- 0.02	-	D(254)*
1	4	0.32 +/- 0.02	Leucine/ Isoleucine	K(vis)*
n.d.	3	0.44 +/- 0.01	Threonine	K(vis)*
n.d.	4	0.51 +/- 0.01	-	I(254); G(366); F(366)*; E(366)*; D(254)*
n.d.	4	0.62 +/- 0.03	Shikimic acid	B(vis)*; J(vis)
4	n.d.	0.54 +/- 0.01	-	C(vis)*; B(vis); A(vis); D(254); J(vis)*
2	1	0.65 +/- 0.00	-	F(366)
4	n.d.	0.83 +/- 0.02	-	C(vis)*; B(vis); A(vis); J(vis)*
4	1	0.96 +/- 0.01	Chlorophyll	A(vis); J(vis); H(366); G(366); F(366)*; K(vis); I(254); C(vis); B(vis); E(366)*; D(254)

Table 4. Biomarkers detected in all the thin layer chromatography systems with the stationary phase: TLC aluminium sheets cellulose plate Merck 1.05552 and the mobile phase 1: 1-butanol:acetic acid:water (4:1:5) (upper phase) and all the derivatization reagents except A and B.

Control VI	Exposed VI	Rf-value	Suggested Compound	Derivatisation Reagents Used for Detection
n.d	4	0.10 +/- 0.01	-	C(vis)*
n.d	4	0.10 +/- 0.01	Lysine	K(vis)*
1	4	0.14 +/- 0.01	Glutamine	K(vis)*
n.d	4	0.27 +/- 0.02	-	C(vis)*; D(366); J(vis)*; F(366)*; E(366)
n.d	4	0.27 +/- 0.02	Proline	K(vis)*
4	1	0.27 +/- 0.01	-	G(254)*; I(366)
1	4	0.41 +/- 0.01	Valine	K(vis)*
1	3	0.50 +/- 0.02	Phenylalanine	K(vis)*
4	1	0.53 +/- 0.03	-	F(366)*; E(366)
1	4	0.57+/- 0.01	Leucine/ Isoleucine	K(vis)
4	1	0.58 +/- 0.03	-	D(366); F(366)*; I(366); E(366);
4	1	0.77 +/- 0.01	-	F(366)*; I(366)
4	n.d.	0.95 +/- 0.02	Chlorophyll	C(vis); G(254)*; D(366); J(vis); F(366)*; I(366); E(366)*; K(vis)

For all the tables, the Visual Intensity (VI) from 0 to 4 for the best reagents (*) is presented for control and exposed plant extracts. Vis = detected in visual light; 254 = detected in UV-light: 254 nm and 366 = detected in UV-light: 366 nm.

All compounds were identified comparing with standards. Knowing the Rf-values, color reactions and UV-spectra the identification was facilitated. A total number of 13, 16, 18, 19 compounds (biomarkers) were detected in ethanolic extracts of either exposed (8, 10, 11 and 12 compounds) or control plants (5, 6, 8 and 9 compounds). Totally, four different biomarker patterns, including glutamine, proline, leucine or isoleucine and chlorophyll, were identified (table 1 to 4) to describe the glyphosate induced respons of *A. spica-venti*. Furthermore, valine, threonine and shikimic acid were detected in two of the biomarker patterns and lysine and phenylalanine were only detected in one of the patterns. For all HPPC systems, only the content of chlorophyll decreased in exposed plants, while the content of all the other of the above mentioned compounds increased. The four biomarker patterns can be used as one biomarker pattern to describe the glyphosate exposure of *A. spica-venti*. However, an overlap of compounds will be seen. Usually more advanced techniques as NMR spectroscopy and gas chromatograph (GC)/mass spectrophotometer (MS) and multivariate analysis for metabolite fingerprinting is used (Ward et al. 2003; Prithiviraj et al. 2004). Ward et al. used crude extracts of ecotypes of *Arabidopsis thaliana* as methods to produce a reproducible metabolite fingerprints, metabolomics, to conclude the identity and relative levels of metabolites differing between samples (Ward et al. 2003). Prithiviraj et al. used headspace GC/MS of volatile metabolites profiling the compounds as metabolomics to distinguish inoculated onion bulbs with three different pathogens (Prithiviraj et al. 2004).

The biomarker proline has previously been described as a compound present at increased concentration following various types of stress exposure in plants. Water stress (Irigoyen et al. 1992), drought stress (Good and Zaplachinski 1994), heavy-metals- (Mehta and Gaur 1999), and NaCl stress (Lee and Liu 1999) induce proline accumulation. The production of valine, leucine and isoleucine are known to be inhibited by sulfonylureas (Chaleff and Mauvais 1984), but an accumulation of these amino acids as well as threonine following exposure to glyphosate has not previously been reported. Our observation that shikimic acid was accumulated after exposure to glyphosate is consistent with previous studies (Pline et al. 2002; Lydon and Duke 1988). The increased content of phenylalanine was unexpected, since glyphosate inhibits the biosynthesis of the aromatic amino acids. A possible explanation could be that more than one pathway for phenylalanine synthesis exists in *A. spica-venti* or that phenylalanine is a metabolite of storage compounds. The unidentified compounds are in progress to be identified. Although some of these compounds have previously been observed following glyphosate exposure, the combination of compounds has not previosly been presented as an overall pattern of changes.

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