Journal of Chromatography, 502 (1990) 201–207
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 113

Note

High-performance liquid chromatographic determination of the herbicide glyphosate and its metabolite (aminomethyl)phosphonic acid and their extraction from cyanobacteria

H. A. POWELL, N. W. KERBY and P. ROWELL*

Agricultural and Food Research Council (AFRC) Research Group on Cyanobacteria, Department of Biological Sciences, University of Dundee, Dundee DD1 4HN (U.K.)

(First received June 13th, 1989; revised manuscript received October 9th, 1989)

Glyphosate[a] [N-(phosphonomethyl)glycine] is a very broad spectrum, post-emergence herbicide. The free acid (PMG) has strong herbicidal properties, although it is the isopropylamine salt (IPA) rather than PMG which is the active ingredient of Roundup® for soil use and of Rodeo® for use in water. Once applied to plants, by foliar spray, it is absorbed and translocated to other plant tissues where its primary effect is to interrupt aromatic amino acid biosynthesis by inhibition of the enzyme 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase[1,2]. Glyphosate may also directly affect the enzyme 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase[3]. Many secondary effects of glyphosate have been described, including increased metabolism of indole-3-acetic acid[4] and increased levels of both hydroxybenzoic acids[5] and phenolic compounds[6].

Glyphosate itself is apparently non-toxic to animals although some toxic effects have been attributed to the non-herbicidal ingredients present in commercial formulations[7]. Residual herbicide in the soil is readily adsorbed to soil particles and subsequently undergoes microbial degradation to ammonia and carbon dioxide[8,9]. The major degradative metabolite of PMG is (aminomethyl)phosphonic acid (AMPA) although degradation via sarcosine and glycine has also been described[10].

A variety of methods for the analysis of glyphosate and AMPA derivatives have been described including gas chromatography[11,12], thin-layer chromatography[13,14] and high-performance liquid chromatography (HPLC) with both pre-column[15–17] and post-column[18,19] derivatisation. Detection of underivatised glyphosate following HPLC has also been described[20]. These methods generally involve the use of extensive, laborious clean-up procedures including ion-exchange chromatography and gel chromatography, which may result in sample loss and reduced reproducibility, especially when no internal standard is used.

[a] Glyphosate is used to describe any form of the herbicide; N-(phosphonomethyl)glycine (PMG), PMG isopropylamine salt (IPA) and Roundup®, are used to specify free acid, IPA salt and commercial formulation, respectively.

0021-9673/90/$03.50

This paper describes a method for the detection of phenylthiocarbamyl (PTC) derivatives of glyphosate and AMPA using the Waters Pico Tag™ amino acid analysis method[21] modified by us for use with a radially compressed column. The method is sensitive, reproducible and rapid and can also be combined with amino acid detection and quantification, giving significant advantages over other previously described methods. A method for sample preparation for the detection of glyphosate and the pool of free amino acids from cyanobacteria is also described.

MATERIALS AND METHODS

Instrumentation

A Waters HPLC system was used, consisting of two Waters 510 pumps, a 10-μl fixed volume sample loop with Rheodyne valve Model 7125 and a valve position sensing switch Model 7161. The column was a Waters Nova-Pak C_{18} (100 mm × 8 mm I.D., 4 μm particle size) Radial-Pak cartridge fitted in an RCM 8 × 10 radial compression module at 17.2 MPa and at ambient temperature. For full physiological amino acid analysis a Waters Pico Tag column (300 mm × 3.9 mm I.D.) was used and the temperature was maintained at 46°C with a column heater–temperature control module. The PTC derivatives were detected using a Waters LC Model 455 variable-wavelength spectrophotometer set at 254 nm. The system was controlled, data collected and analysed using a System Interface Module, a NEC APCIV computer and Waters Maxima 820 software. Samples were prepared using a Waters Pico Tag work station with a Trivac D16B pump. Fractions from samples containing ^{14}C-labelled PMG were collected with a Pharmacia Frac100 fraction collector and counted on a Packard Tricarb 4000 scintillation counter.

Sample preparation and derivatisation

Samples of up to 200 μl were dispensed into Corning sample tubes, 50 mm × 6 mm I.D., and placed in a reaction vial with a capacity of 14 tubes. Using a Pico Tag workstation, samples were dried under vacuum to less than 9.3 Pa and then resuspended in 10 μl of a mixture of methanol–1 *M* sodium acetate–triethylamine (2:2:1, v/v/v) and redried to less than 9.3 Pa. A 20-μl aliquot of reaction mix which consisted of methanol–phenylisothiocyanate (PITC)–triethylamine–water (7:1:1:1, v/v/v/v) was added to each sample. Following a 20-min incubation at room temperature, the samples were redried to less than 9.3 Pa. For analysis, samples were resuspended in 100 μl of loading buffer [5%, (v/v) acetonitrile in 5 m*M* Na_2HPO_4, pH 7.4], and centrifuged at 10 000 *g* for 1 min prior to injection.

Mobile phase

Eluents A and B consisted of 2.5% (v/v) acetonitrile in 70 m*M* sodium acetate trihydrate pH 6.45 and acetonitrile–water–methanol (45:40:15, v/v/v), respectively. The eluents were filtered through a 0.22-μm Durapore (Millipore) membrane filter and degassed by sonication in a Cole–Parmer sonicating water bath before use. All reagents were of HPLC grade and Milli-Q (Millipore) water was used throughout. National Diagnostics Ecoscint A was used for liquid scintillation counting.

Glyphosate determination

In order to determine the retention times and linearity of the peak response to glyphosate and AMPA derivatives, samples were prepared such that between 0.05 and 10 nmol of PMG, IPA, commercial Roundup or AMPA were applied to the Radial-Pak™ column. Each sample also contained 1 nmol norleucine or taurine as an internal standard. The retention times of other possible metabolites of glyphosate, namely sarcosine and glycine, were also determined. The identity of the PTC–glyphosate peak was verified using N-phosphono-[^{14}C]methylglycine (^{14}C-PMG). Fractions eluting from the column were collected for liquid scintillation counting.

Preparation of cyanobacterial samples

Synechocystis PCC 6803 cultures were grown in 100 ml of BG11 medium[22], on an orbital shaker, at room temperature and a photon fluence rate of 30–50 μmol m^{-2} s^{-1}. Chlorophyll *a* was determined by the method of Mackinney[23] following methanol extraction of cells. Cells from cultures containing between 1 and 20 μg chlorophyll *a* per ml, were harvested by centrifugation at 10 000 *g* for 10 min and were washed twice by resuspension of the cell pellet in BG11. The cell pellet was then resuspended in 200 μl of 70% (v/v) ethanol acidified to pH 3 with hydrochloric acid, and extracted at −20°C for at least 12 h. The internal standard was added to the sample during the ethanol extraction. Extracts were centrifuged at 10 000 *g* for 10 min and then prepared for HPLC analysis as described above. In order to determine the recovery of glyphosate, samples from glyphosate-free cultures of the cyanobacterium *Synechocystis* PCC 6803 were fortified with 2–20 nmol of glyphosate (as Roundup) or AMPA, and prepared such that 0.1–1 nmol was applied to the column, assuming a recovery of 100%.

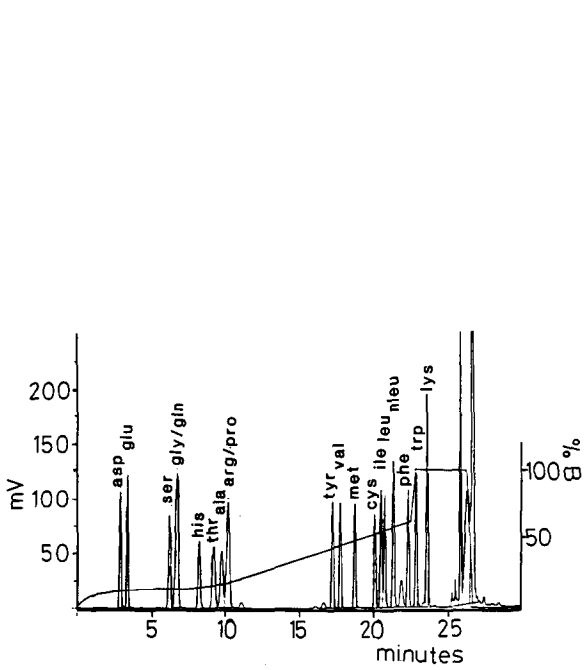
Chemicals

All chemicals were of the highest grade available and were purchased from BDH (Poole, U.K.), Millipore (Watford, U.K.) (for Waters products), Sigma (Poole, U.K.) or Rathburn Chemicals (Walkerburn, U.K.). Glyphosate (96.7% pure) andIPA (62% pure) were gifts from Monsanto (St. Louis, MO, U.S.A.). Roundup which contains 36% (w/v) glyphosate was obtained from a farm supplier. ^{14}C-PMG was purchased from Amersham International (Amersham, U.K.).

RESULTS AND DISCUSSION

Peak identification

Fig. 1 shows the gradient profile of percentage of eluent B developed for the maximal separation of Pierce H standard amino acids supplemented with norleucine, glutamine and tryptophan on the Radial-Pak column. This gradient was used for the analysis of glyphosate combined with analysis of the pools of free amino acids. HPLC elution profiles of samples containing 1 nmol of PMG, IPA or Roundup and 1 nmol of norleucine are shown in Fig. 2, together with the elution profile of ^{14}C-PMG. From the ^{14}C-labelling pattern, peak A was identified as PMG. Peak B, which has an area of up to 0.5% of that of peak A, coeluted with AMPA while peak C, with an area of 3% of that of peak A did not coelute with either glycine or sarcosine. As the retention times obtained by reversed-phase chromatography of similar compounds



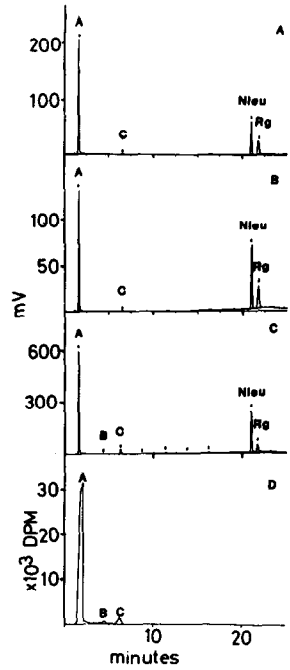


Fig. 1. Elution profile of PTC derivatives of Pierce H standard amino acids supplemented with norleucine (nleu), glutamine and tryptophan (1 nmol of each) from the Radial-Pak column using a 30-min run time. The profile of percentage of eluent B used for separation is also shown.

Fig. 2. Elution profiles of PTC derivatives of glyphosate and norleucine (1 nmol of each) from the Radial-Pak column using a 30-min run time. (A) PMG and norleucine. (B) IPA and norleucine. (C) Roundup and norleucine. (D) Profile of DPM recovered from 0.04 μCi ^{14}C-PMG. Peaks A, B and C have retention times of 1.6, 4.3 and 6.3 min, respectively. Rg denotes a reagent peak. Nleu = Norleucine.

are dependent primarily on their polarity, it can be argued that peak C was N-methylaminomethylphosphonic acid (MAMPA). MAMPA is more polar than glycine, having a phosphonic acid residue rather than a carboxylic acid residue and less polar than AMPA, having an extra methyl group. Peak C, tentatively identified as MAMPA, also contains a small fraction of the ^{14}C label from the ^{14}C-PMG. Peaks B and C were probably breakdown products of glyphosate which may have been formed during the derivatisation reaction. The major product of glyphosate degradation is AMPA although MAMPA has also been found[8] at much lower levels than AMPA. Extra peaks detected on analysis of the Roundup sample (Fig. 2C) may have been due to other UV absorbing or PTC derivatising compounds in the formulation.

As Fig. 2 shows, PMG had a short retention time, resulting in the separation of the herbicide from other components in physiological samples (Fig. 3). Any interference by other amino acids, which might lead to inaccuracies in the quantification of glyphosate was, therefore, minimised.

Response and recovery

Over the range of 0.05 to 10 nmol, for PMG (peak A) from PMG, IPA and Roundup, and with both the Radial-Pak cartridge and the Pico Tag column, a linear

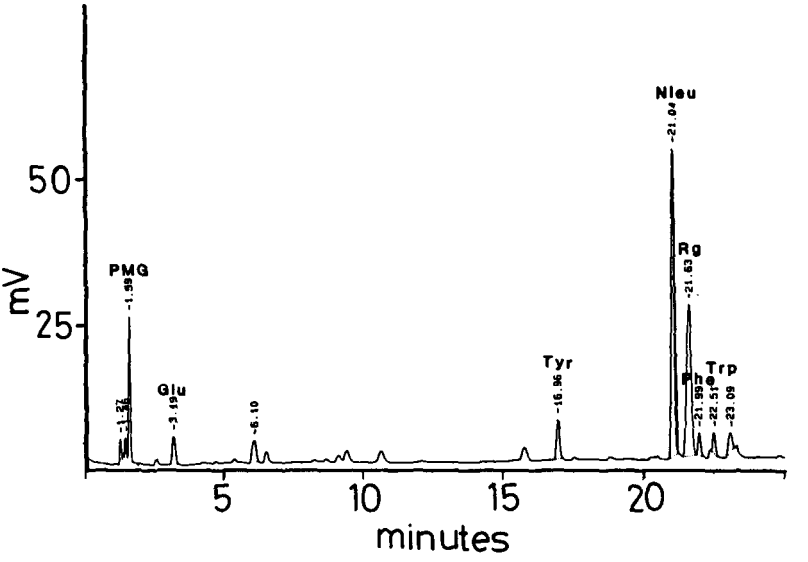


Fig. 3. Elution profiles of PTC derivatives of glyphosate and free-pool amino acids extracted from *Synechocystis* PCC 6803 grown in BG11 in the presence of 10 m*M* IPA and 1 m*M* tyrosine, phenylalanine and tryptophan. Rg denotes a reagent peak. Nleu = Norleucine.

response to the amount of PMG in the sample was obtained. Responses were calculated relative to 1 nmol norleucine. When data from all sources were collated, the same response (85.8 ± 5.5%) relative to the response for norleucine was obtained, showing no difference between either the various sources of glyphosate or between the two columns (Table I). Samples containing AMPA also gave a linear response of 59 ± 3% relative to the response for norleucine which was different to that obtained for PMG.

The high levels of reproducibility obtained were facilitated by the use of an internal standard which, when added during extraction, corrected for variation in the efficiency of derivatisation, sample preparation and injection. The sensitivity and

TABLE I

LINEARITY OF RESPONSE TO PTC–PMG AND PTC–AMPA FOLLOWING SEPARATION ON PICO TAG AND RADIAL-PAK COLUMNS

Samples containing between 0.05 and 10 nmol of glyphosate were applied to the columns.

Compound	*Column*	*Number of samples*	*Correlation coefficient*
PMG	Pico Tag	10	0.997
PMG	Radial-Pak	7	0.998
IPA	Pico Tag	6	0.995
IPA	Radial-Pak	8	0.998
Roundup	Radial-Pak	5	0.996
Collated data (above)		36	0.994
AMPA	Radial-Pak	7	0.986

reproducibility of this method may allow its use for the accurate determination of picomol levels of glyphosate, it may be especially useful when analysis of other amino acids is also required. A 10-μl water sample containing 1 ppm of glyphosate could readily be quantified.

Recovery of glyphosate from fortification experiments was similar to, or greater than that obtained by other methods. Recovery of PMG from five samples of the cyanobacterium *Synechocystis* PCC 6803 fortified with Roundup was 78 ± 8.49% and the recovery of AMPA from six fortified samples of the cyanobacterium *Synechocystis* PCC 6803 was 77 ± 12.6%. These values compare favourably with those obtained for fortified blackberries (83 ± 14%)[18], asparagus (89 ± 36%) and kiwi fruit (87 ± 7.1%)[24].

The method of sample preparation developed for cyanobacteria, which did not require deproteination prior to analysis, should be easily adaptable for use with other tissues of both plant and animal origin. A simple maceration step prior to ethanol extraction may be required for such tissues.

Rapid analysis

The method described above allowed the determination of glyphosate as well as other amino acids which may be of use in studies of herbicide action. A more rapid analytical method can be used if other amino acid data are not required. An analysis time of 15 min including column reequilibration is possible with taurine (retention time 6.1 min) used as the internal standard (Fig. 4). The response relative to taurine was linear with a correlation coefficient of 0.994 (n = 10) over the range 0.1 to 10 nmol of glyphosate.

Sample storage

Samples were stable during long-term storage, at least three months, in 70%

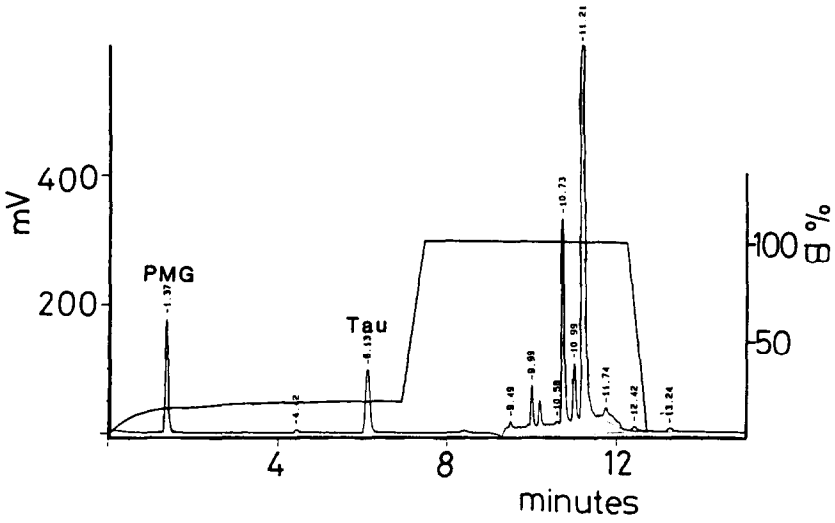


Fig. 4. Elution profile of PTC–glyphosate and PTC–taurine from the Radial-Pak column using a 15-min run time showing the gradient profile of eluent B percentage.

(v/v) acidified ethanol at −20°C prior to vacuum desiccation. Once dried and treated with drying solution, samples could be stored for at least five days under vacuum or nitrogen at −20°C. Following derivatisation samples are only stable for 24 h under vacuum at −20°C.

The above method of PMG and AMPA analysis is sensitive, reproducible and rapid. In contrast to some previously published methods, sample preparation was accomplished with relative ease. Up to 14 samples were prepared simultaneously and their stability prior to derivatisation allowed them to be prepared in advance. Analysis of 30 samples a day was achieved and the use of an autosampler would allow the analysis of even greater numbers each day.

REFERENCES

1 J. L. Rubin, C. G. Gaines and R. A. Jensen, *Plant Physiol.*, 70 (1982) 833–839.
2 H. Hollander and N. Amrhein, *Plant Physiol.*, 66 (1980) 823–829.
3 R. J. Ganson and R. A. Jensen, *Arch. Biochem. Biophys.*, 260,(1988) 85–93.
4 T. T. Lee, *Physiol. Plant.*, 54 (1982) 289–294.
5 J. Lydon and S. O. Duke, *J. Agric. Food Chem.*, 36 (1988) 813–818.
6 S. O. Duke and R. E. Hoagland, in E. Grossbard and D. Atkinson (Editors), *The Herbicide Glyphosate*, Butterworths, Woburn, MA, 1985, pp. 75–79.
7 Y. Sawada, Y. Nagai, M. Ueyama and I. Yamamoto, *Lancet*, No. 8580 (1988) 299.
8 M. L. Rueppel, B. B. Brightwell, J. Schaefer and J. T. Marvel, *J. Agric. Food Chem.*, 25 (1977) 517–528.
9 L. Torstennson, in E. Grossbard and D. Atkinson (Editors), *The Herbicide Glyphosate*, Butterworths, Woburn, MA, 1985, pp. 137–150.
10 G. M. Kishore and G. S. Jacob, *J. Biol. Chem.*, 262 (1987) 12164–12168.
11 R. A. Guinivan, N. P. Thompson and W. B. Wheeler, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 35–39.
12 H. A. Moye and C. L. Deyrup, *J. Agric. Food Chem.*, 32 (1984) 192–195.
13 P. Sprankle, C. L. Sandberg, W. F. Meggitt and D. Penner, *Weed Sci.*, 26 (1978) 673–674.
14 J. C. Young, S. U. Khan and P. B. Marriage, *J. Agric. Food Chem.*, 25 (1977) 918–922.
15 L. N. Lundgren, *J. Agric. Food Chem.*, 34 (1986) 535–538.
16 C. J. Miles, L. R. Wallace and H. A. Moye, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 458–461.
17 C. J. Miles and H. A. Moye, *J. Agric. Food Chem.*, 36 (1988) 486–491.
18 T. E. Archer and J. D. Stokes, *J. Agric. Food Chem.*, 32 (1984) 586–588.
19 H. A. Moye, C. J. Miles and S. J. Scherer, *J. Agric. Food Chem.*, 31 (1983) 69–72.
20 A. J. Burns, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 1214–1219.
21 R. L. Heinrickson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65–74.
22 R. Rippka, J. Deruelles, J. B. Waterbury, M. Herdman and R. Y. Stanier, *J. Gen. Microbiol.*, 111 (1979) 1–61.
23 G. Mackinney, *J. Biol. Chem.*, 140 (1941) 315–322.
24 J. N. Seiber, M. M. McChesney, R. Kon and R. A. Leavitt, *J. Agric. Food Chem.*, 32 (1984) 678–681.