ELSEVIER

Journal of Chromatography A, 795 (1998) 53–60

JOURNAL OF CHROMATOGRAPHY A

New and simple method for the analysis of the glutathione adduct of atrazine

Rita Frassanito[a,*], Massimo Rossi[a], Luana K. Dragani[a], Carlo Tallarico[b], Antonio Longo[b], Domenico Rotilio[a]

[a] *"G. Paone" Environmental Health Center, Istituto di Ricerche Farmacologiche "Mario Negri", Consorzio Mario Negri Sud, 66030 S. Maria Imbaro, Italy*

[b] *Pharmacokinetic and Metabolism Laboratory, Sigma Tau S.p.A., 00040 Pomezia, Italy*

Abstract

A specific and simple protocol for the preparation, separation, identification and quantitation of glutathione (GSH) conjugates is often not available, despite the role of these compounds as an important pathway of contaminant transformation. Here, a new method is reported for the analysis of the glutathione adduct of atrazine (GSHA) in bacterial samples. The compound was synthesised by modifying a previously described procedure, isolated by Isolute-ENV$^+$ solid-phase extraction (SPE) columns containing crosslinked polymer (styrene–divinylbenzene) phase, identified and quantified by capillary zone electrophoresis with UV detection and characterised by electrospray ionisation mass spectrometry. A very good recovery on bacterial samples was obtained by the same SPE method used for the preparation of the standard compound. © 1998 Elsevier Science B.V.

Keywords: Atrazine; Glutathione derivatives; Pesticides

1. Introduction

Enzymatically mediated conjugation reactions between electrophilic contaminants and biological nucleophiles (thiols) result in the formation of unanticipated and difficult-to-detect metabolites. Conjugation reactions with glutathione (GSH) have been suggested as an important pathway of contaminant transformation, particularly in the pesticide literature [1], playing a major role in plant resistance to herbicides [2]. Moreover, research must be focused on supporting the hypothesis that glutathione conjugation is a potentially important environmental phenomenon involving multiple organisms, and that plants and microorganisms are competent in glutathione conjugation for phytoremediation and bioremediation [3,4].

Glutathione-S-transferase (GST) catalyses the nucleophilic conjugation of GSH with many diverse electrophilic substrates [5]. Although the role of GSH conjugation, whether it involves GST or not, in the detoxification–degradation of xenobiotics by terrestrial microorganisms has been postulated [6–10], it is necessary to elucidate the role of this mechanism in the biodegradation of xenobiotic compounds.

Moreover, relatively little information is available on the extent to which GSH conjugation in plants and microorganisms affect the mass balance of contaminants in the environment, often because a specific and simple protocol for the preparation,

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01041-8

separation and identification and quantitation of these metabolites is not available.

Lau et al. [11] surveyed GST distribution in a wide range of microorganisms. GST activity occurred in 50% of the bacterial strains tested with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Lower GST activity occurred in bacteria compared with fungi, algae and protozoa [11]. Recently, investigations of the role of bacterial GST in the degradation of the herbicide alachlor and preliminary studies of the metabolism of CDNB- and alachlor–glutathione conjugates have been undertaken [6]. There are no studies about the isolation of GSH adducts in pesticide-degrading bacteria.

s-Triazine herbicides are used to control grasses and broadleaf weeds in a variety of crops; in particular, atrazine is a commercially important herbicide that is used for weed control in corn.

The dominant phase I metabolic reaction in plants and mammals seems to be a cytochrome P450-mediated N-dealkylation, while the primary phase II reaction is the GST-catalysed conjugation with GSH. In higher plants, it may proceed enzymatically, mediated by GST enzymes, or non-enzymatically [12–15], thus forming non-phytotoxic compounds. A report on insect and fish species exposed to atrazine shows the formation of a water-soluble metabolite and the presence of GST isoenzymes [16].

Numerous studies on the environmental fate of atrazine show that it is transformed slowly [17,18]. Recently, microorganisms capable of mineralizing high concentrations of atrazine and of rapidly transforming it into hydroxyatrazine have been isolated. Dechlorination seems to be the critical step in atrazine degradation [19,20]. Several microbial metabolites of this herbicide have been isolated and analysed by different methods [21–23]

The GSH conjugate of atrazine (GSHA) in plants has been detected by reversed-phase high-performance liquid chromatography (HPLC) with UV detection (unlabelled atrazine) [13] or with a radioactivity monitor (^{14}C-labelled atrazine) and identified by fast atom bombardment mass spectrometry (FAB MS) [24], often using extraction by C_{18} phase columns without reporting the recovery. The chromatographic methods require large sample volumes and elaborate sample preparation.

Capillary electrophoresis (CE) has become an important separation technique that shows a very high resolution power. Detection of GSH and its derivatives has been performed in the capillary isotachophoretic mode [25,26], mainly to quantify GST activity. CE does not permit high accuracy in compound quantitation and it has difficulty in discriminating the compounds of interest from interferences; this is a disadvantage for the analysis of biological matrices. CE has the right features to resolve these problems by focusing attention on the selected analyte.

Reduced and oxidised forms of GSH have been determined in mammalian red blood cells [27], in rat liver extracts [28] and in human erythrocytes [29] with CE methods, but, as far as we know, this analytical technique has never been applied to the determination of GSH conjugates of biological interest. Moreover, CE methods have been applied to the analysis of *s*-triazine herbicides and their metabolites, by capillary zone electrophoresis (CZE) [30,31] and micellar electrokinetic capillary chromatography [32]; but in this case also, conjugate compounds of biological interest have never been taken into consideration.

Here, we report a new method for the analysis of the glutathione adduct of atrazine by simple solid-phase extraction (SPE) and CE. The structure of the adduct was confirmed by electrospray ionisation mass spectrometry (ESI-MS).

2. Experimental

2.1. Chemicals

Atrazine (97.9%) was purchased from Labor Dr Ehrenstonfer (Ausburg, Germany), the reduced form of glutathione was obtained from Sigma (St. Louis, MO, USA), trimethylamine (RPE grade), sodium carbonate, calcium chloride, phosphoric acid, sodium dihydrogen phosphate, sodium hydroxide, hydrochloric acid, acetone, dichloromethane and methanol were purchased from Carlo Erba (Milan, Italy). Each solvent was of RPE grade.

2.2. Synthesis and isolation of the atrazine adduct

The glutathione–atrazine adduct was synthesized

using a slight modification of a previously described method [33,34]. Atrazine was dissolved in acetone, to which an excess of trimethylamine was added. This solution was evaporated to dryness after 18 h at room temperature under stirring. An equimolar amount of reduced glutathione and sodium carbonate in aqueous solution was added to dissolve the trimethylamino derivative. The solution was heated to 50°C, stirred for 18 h, diluted with 0.01 *M* $CaCl_2$, and partitioned twice with dichloromethane. The aqueous solution was concentrated in vacuo to a few millilitres and adjusted to pH 2 with 1 *M* HCl. We used Isolute Env^+ columns containing 1 g of phase to isolate the standard GSHA after its preparation. This column, produced by IST (Hengoed, UK) and obtained from Stepbio (Bologna, Italy), is a high-capacity, highly crosslinked, polystyrene-based polymer column. It was conditioned first with methanol, then with a 0.01-*M* HCl solution. The sample was loaded using very slow flow-rates (with no pressure), salt and glutathione interferences were minimised by elution with 0.01 *M* HCl solution, and with acetone to purify the adduct from atrazine residues. Then the column was dried under nitrogen flow and the adduct was eluted using methanol–acetic acid (95:5, v/v). The standard compound was obtained in the solid state by drying the eluate. The yield was calculated as the mean value of four samples after the extractive procedure (32.2±1.3%, $n=4$).

2.3. Thin-layer chromatographic analysis

Thin-layer chromatography (TLC) was performed on 250 silica F-PA (19 C) gel plates purchased from Baker (Phillipsburg, NJ, USA) using *n*-butanol–acetic acid–water (11:5:4, v/v/v) as the developing solvent. Compound was located by viewing it under UV light and by spraying with ninhydrin.

2.4. CE analysis

Capillary electrophoresis was performed using a P/ACE 2100 system (Beckman Instruments, Fullerton, CA, USA), equipped with a UV detector, and elaborated with a GOLD 6.0 software acquisition system on an IBM 55SX personal computer. The column used was an uncoated fused-silica capillary tube (Beckman) of 75 μm I.D., 375 μm O.D., 40 cm length to detector and with a total length of 47 cm. Electrophoretic analysis was performed using the UV detector set at a wavelength of 230 nm, and at an applied voltage of 15 kV at 20°C. Moreover, the silica column was pre-rinsed with bidistilled water (1 min) and separation buffer (1.5 min); between runs, the capillary was washed with 1 *M* NaOH (2 min) and water (1 min). Samples were then hydrodynamically injected at $3.45 \cdot 10^3$ Pa for 7 s, for a total volume of 45 nl. The electrolyte buffer was obtained by mixing a sodium dihydrogenphosphate solution (50 m*M*) with a phosphoric acid solution (50 m*M*) to reach the desired pH value. All solutions were prepared with deionized water (Milli-Q, Millipore, Bedford, MA, USA) and filtered using 0.2 μm Minisart filters from Sartorius (Göttingen, Germany). The separation efficiency was measured by the number of theoretical plates (N) according to the formula: $N=5.54\ (t_R/w)^2$, where t_R is the retention time of a compound and w is the peak width at half-peak height [35]. Calibration curves were obtained by hydrodynamic injection of concentrations, from 0.2 to 10 μg/ml, of our compound for 7 s, at a pressure of $3.45 \cdot 10^3$ Pa.

2.5. MS analysis

All mass spectra were obtained with an API-III triple quadrupole mass spectrometer (Perkin-Elmer/SCIEX, Thornhill, Canada), equipped with a nebulization-assisted electrospray source (ion spray) in the positive ion mode. The ion spray needle was kept at a potential of 5.8 kV, with an orifice voltage of 80 V, a nebulizer pressure of $3.4 \cdot 10^5$ Pa, and a nitrogen curtain gas flow-rate of 1.8 l/min. The sample was directly injected into the mass spectrometer by infusion through the API interface.

2.6. Preparation of bacterial samples

Gram-negative bacterial strains, collected from soil, were cultured aerobically for 18 h at 37°C in Luria-Bertani (LB) medium. Cells were washed with 10 m*M* potassium phosphate buffer and disrupted by sonication (five bursts of 1 min each, at 50 W and 20 kHz) with a ''Vibra cell'' Vc50 (Sonics and Materials, Danbury, CT, USA) sonicator. The particulate matter was removed by centrifugation at 10 000 *g*

for 30 min and the supernatant was inactivated by boiling for 3 min.

2.7. Recovery assay

The recovery test was performed in quadruplicate by spiking 500 μl of bacterial extract with 100 μl of the appropriate amounts of GSHA. The fortification levels used were 20, 5, 1 and 0.4 μg/ml. The bacterial samples were adjusted to pH 2 with 1 *M* HCl and extracted using the same procedure as described above, using columns containing 200 mg of phase. After elution by methanol–acetic acid (95:5, v/v), the samples were dried under nitrogen flow and dissolved in 1 ml of distilled water just before processing.

3. Results and discussion

3.1. Isolation of the glutathione–atrazine standard conjugate

The preparation and isolation of the GSHA standard adduct yields a product that is stable either in the solid state or in a concentrated aqueous solution at 4°C. The isolation step is easy and reproducible, as it does not require elaborate sample preparation. The purity, after purification by SPE performed with Isolute-ENV$^+$, was tested by TLC (data not shown), by the electrophoretic- and the mass spectral data.

3.2. CZE analysis

Fig. 1 shows the electropherogram of the glutathione–atrazine adduct. We have obtained a good peak shape ($N \approx 130\,000$) with a migration time of less than 15 min, making the analysis very rapid.

Under the previously described conditions, residual atrazine and glutathione are not detectable and they cannot interfere (data not shown). As is known, pH is an important parameter in CZE, therefore,

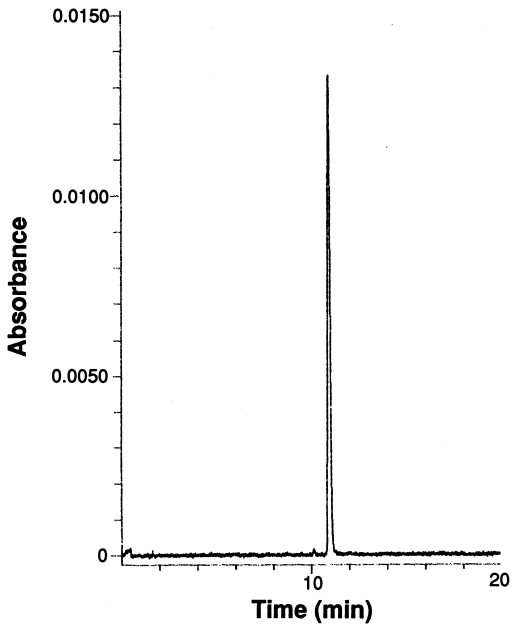

Fig. 1. CZE analysis of GSHA (50 m*M* phosphate buffer, pH 2; 15 kV; 20°C; 230 nm).

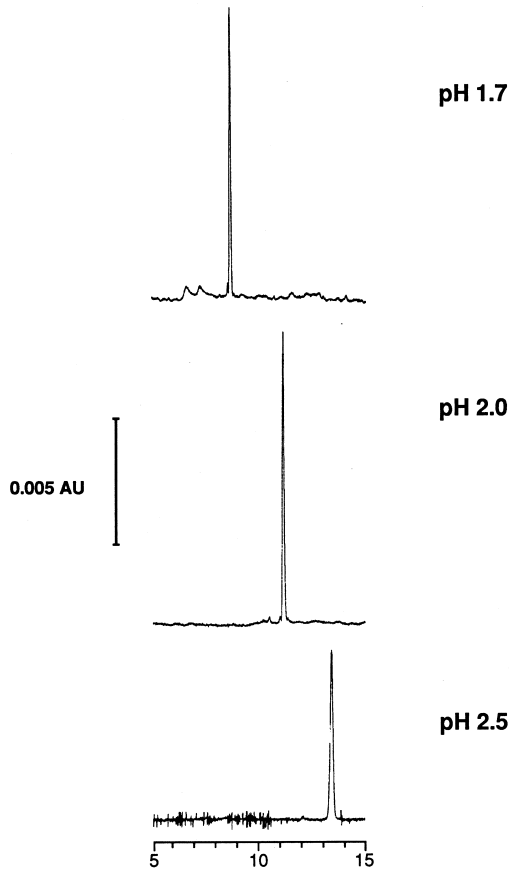

Fig. 2. Electropherograms of GSHA performed using separation buffer at different pH values. The other conditions are described in Fig. 1.

three different electropherograms at different pH values were performed, as shown in Fig. 2. The GSHA adduct was analysed using phosphate buffer at pH values of 1.7, 2.0 and 2.5; in all samples, the phosphate buffer was maintained at a concentration of 50 m*M*. Better separations of the analyte were obtained at pH values of 1.7 and 2.0. Analysis was performed at pH 2.0, because a better baseline was obtained and the capillary column was less stressed, although separation at pH 1.7 is faster. To evaluate the reproducibility of our method, repeated analyses ($n=10$) of GSHA adduct were conducted, obtaining acceptable precision in terms of migration times (R.S.D.≈1%) and peak areas (R.S.D≈5%). The calibration curves used for the quantitation of the GSHA adduct were linear in the range of sample concentrations between 0.2 and 100 μg/ml ($y=4.92\cdot 10^{-2}x+7.58\cdot 10^{-3}$; $R^2=0.998$).

3.3. Mass spectral analysis

The mass spectral analyses of atrazine, glutathione and GSHA are shown in Figs. 3–5, respectively. Each spectrum is dominated by singly or multiply protonated molecular ions, and alkali ion adducts are also present. The mass spectrum of atrazine (Fig. 3) gives two major peaks at m/z 216 and 218, corresponding to M+H and M+3H, respectively. The

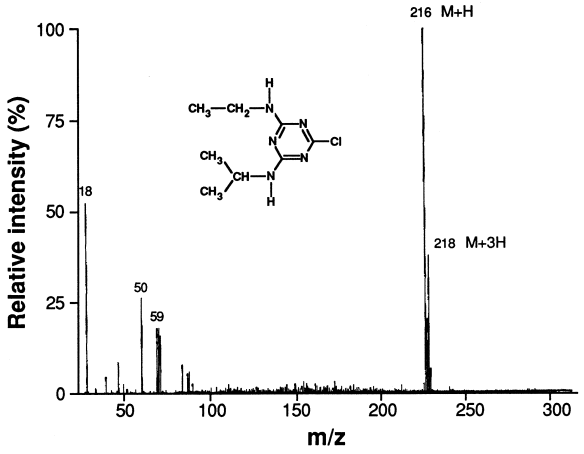

Fig. 3. ESI mass spectrum of atrazine. The spectrum was recorded with a single quadrupole mass spectrometer equipped with a nebulization-assisted electrospray source (ion spray) in the positive ion mode.

GSH spectrum (Fig. 4) shows two peaks at m/z 308 and 330, corresponding to the protonated molecular ion and to the alkali ion adduct (M+Na), respectively. Besides showing its protonated molecular ion (m/z 487), the mass spectrum of GSHA (Fig. 5) has a particular peak at m/z 666, which is probably due to the attachment of an atrazine-derived fragment to the molecular ion ($M+C_8H_{14}N_5$). The peak at m/z 974 is assignable to the doubly protonated dimeric compound (2M+2H).

The high purity of the standard compound obtained by the isolation procedure can be easily seen in the GSHA mass spectrum. Both electrophoresis and MS techniques allow us to exclude the presence of possible degradation products.

3.4. Recovery

Extraction with the Isolute-ENV$^+$ phase (previously used with medium-to-highly polar compounds) was tested by conditioning the sample at two different pH values (Fig. 6). The electropherogram at pH 2 shows only one peak and a good signal-to-noise ratio. The differences between the two electropherograms are due to the extractive, and not to the instrumental, conditions, in that the injected samples have the same final pH values. The choice of pH 2 as the conditioning parameter gives a very good recovery for bacterial samples (Table 1), being a very simple procedure with fast sample preparation.

This method can be used to determine adduct concentrations in bacterial samples of between 20 and 0.4 μg/ml, where the lowest value corresponds to the LOD.

4. Conclusions

Here, we have presented a simple protocol for the isolation, identification and quantitation of GSH–atrazine conjugate. The standard compound was isolated using an Isolute-ENV$^+$ SPE column, identified and quantified by CZE with UV detection and characterised by ESI-MS. Very good recovery was obtained for bacterial samples using the same SPE method for the preparation of the standard compound.

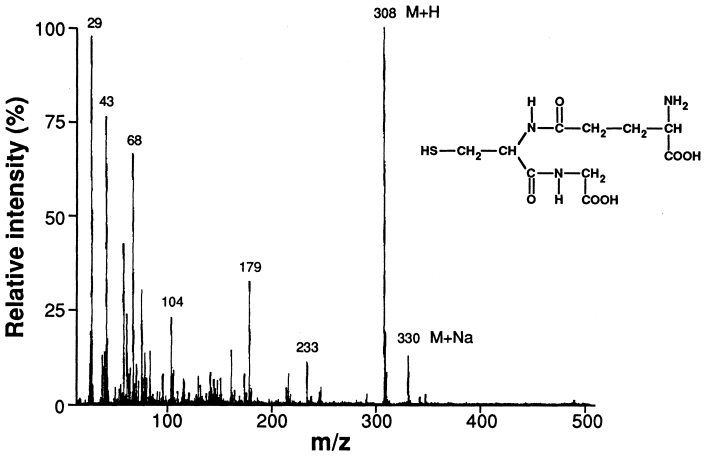

Fig. 4. ESI mass spectrum of glutathione. The conditions are the same as in Fig. 3.

CE is suitable for determining compounds that are otherwise undetectable by classical methods (HPLC and GC). On the other hand, its versatility allows one to perform faster and simpler analyses than the above-mentioned techniques, as shown in our work. The use of an efficient method of recovery allowed us to separate our compound in a quantitative manner and in a form that was free from possible interferences. By testing our method on bacterial samples, we have demonstrated its applicability to biological and environmental matrices, achieving a good level of sensitivity. It could contribute to studying the role of glutathione conjugation as a relevant process involving different organisms in phytoremediation and bioremediation.

Acknowledgements

The authors wish to thank R. Bertazzi for her expert assistance in the preparation of figures and D. Spadano, M.G. Mencuccini, P. Di Nardo and M.P. De Simone for helping to prepare the manuscript. M.

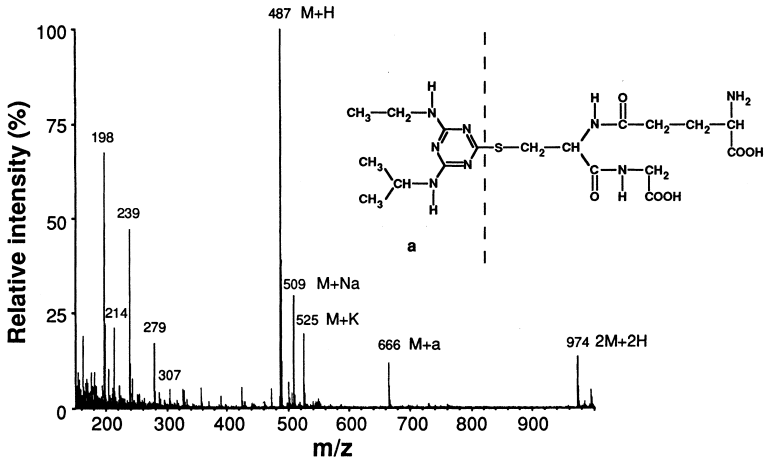

Fig. 5. ESI mass spectrum of GSHA. The conditions are the same as in Fig. 3.

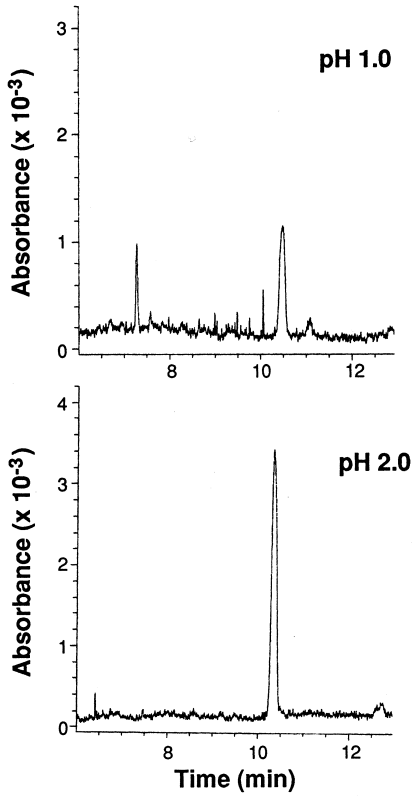

Fig. 6. CZE analysis of GSHA extracted by SPE after conditioning the samples at different pH values.

Rossi and L. K. Dragani are the recipients of a fellowship from the Regional Council of Abruzzo (Esperto in Metodologia della Ricerca). This work has been partially supported by the Italian National Research Council (Convenzione C.N.R.-Consorzio Mario Negri Sud) and by the Regione Abruzzo (POM 1994/1996-Sottoprogramma 3-Misura 3.1-Ricerca e Sperimentazione).

Table 1
Recovery values[a] from fortified bacterial samples

GSHA concentration (μg/ml)	SPE recovery (%)
0.40	93.8±4.0
1.0	97.1±4.2
5.0	85.3±3.5
20.0	93.5±3.8

[a]Errors are standard deviations based on quadruplicate measurements.
SPE conditions: Sample volume=600 μl, pH=2, elution solvent=CH_3OH–CH_3COOH (95:5, v/v).

References

[1] D.H. Hutson, in: D.D. Kaufman, G.G. Still, G.D. Paulson, S.K. Bandal (Eds.), Bound and Conjugated Pesticide Residues (ACS Symposium Series, No. 29), American Chemical Society, Washington, DC, 1976, p.103.
[2] G.L. Lamoureux, D.G. Rusness, in: G.D. Paulson, J. Caldwell, D.H. Hutson, J.J. Menn (Eds.), Conjugation Chemistry (ACS Symposium Series, No. 299), American Chemical Society, Washington, DC, 1986, p. 62.
[3] T.A. Anderson, E.A. Guthrie, B.T. Walton, Env. Sci. Technol. 27 (1993) 2630.
[4] G.A. Buyanovsky, R.J. Kremer, A.M. Gajda, H.V. Kazemi, Bull. Environ. Contam. Toxicol. 55 (1995) 689.
[5] W.H. Habig, M.J. Pabst, W.B. Jakoby, J. Biol. Chem. 249 (1974) 7130.
[6] R.M. Zablotowicz, R.E. Hoagland, M.A. Locke, W.J. Hickey, Appl. Environ. Microbiol. 61 (1995) 1054.
[7] J.A. Field, E.M. Thurman, Environ. Sci. Technol. 30 (1996) 1413.
[8] C.S. Orser, J. Dutton, C. Lange, P. Jablonski, L. Xun, M. Hargis, J. Bacteriol. 175 (1993) 2640.
[9] D. Sheehan, J.P. Casey, Comp. Biochem. Physiol. 104B (1993) 1.
[10] P.C.C. Feng, Pestic. Biochem. Physiol. 40 (1991) 136.
[11] E.P. Lau, L. Niswander, D. Watson, R.R. Fall, Chemosphere 9 (1980) 565.
[12] D.S. Frear, H.R. Swanson, Phytochemistry 9 (1970) 2123.
[13] G.L. Lamoureaux, L.E. Stafford, R.H. Shimabukuro, J. Agric. Food Chem. 20 (1972) 1004.
[14] P.J. Hatton, D. Dixon, D.J. Cole, R. Edwards, Pestic. Sci. 46 (1996) 267.
[15] I. Jablonkai, K. Hatzios, J. Agric. Food Chem. 41 (1993) 1736.
[16] E. Egaas, J.U. Skaare, N.O. Svendsen, M. Sandvik, J.G. Falls, W.C. Dauterman, T.K. Collier, J. Netland, Comp. Biochem. Physiol. 106C (1993) 141.
[17] D.D. Kaufman, P.C. Kearney, Residue Reviews 32 (1970) 235.
[18] R.M. Behki, S.U. Khan, J. Agric. Food Chem. 34 (1986) 746.
[19] R.T. Mandelbaum, L.P. Wackett, D.L. Allan, Appl. Environ. Microbiol. 59 (1993) 1695.
[20] R.T. Mandelbaum, L.P. Wackett, D.L. Allan, Environ. Sci. Technol. 27 (1993) 1943.
[21] S. Papilloud, W. Haerdi, S. Chiron, D. Barcelo', Environ. Sci. Technol. 30 (1996) 1822.
[22] J.R. Pardue, J. AOAC Int. 78 (1995) 856.
[23] R. Frassanito, G. De Socio, D. Laura, D. Rotilio, J. Agric. Food Chem. 44 (1996) 2282.

[24] G.L. Lamoureux, D.G. Rusness, Pestic. Biochem. Physiol. 26 (1986) 323.
[25] C.J. Holloway, R.V. Battersby, Electrophoresis 7 (1986) 304.
[26] D. Tsikas, G. Brunner, J. Chromatogr. 470 (1989) 191.
[27] G. Piccoli, M. Fiorani, B. Biagiarelli, F. Palma, L. Potenza, A. Amicucci, V. Stocchi, J. Chromatogr. A 676 (1994) 239.
[28] P. Pascual, E. Martinez-Lara, J.A. Barcena, J. Lopez-Barea, F. Toribio, J. Chromatogr. 581 (1992) 49.
[29] J. Russell, D.L. Rabenstein, Anal. Biochem. 242 (1996) 136.
[30] F. Foret, V. Sustacek, P. Bocek, Electrophoresis 11 (1990) 95.
[31] Ph. Schmitt, A.W. Garrison, D. Freitag, A. Kettrup, J. Chromatogr. A 723 (1996) 169.
[32] F.M. Lanças, M.A. Ruggiero, Proceedings of the 19th International Symposium on Capillary Chromatography and Electrophoresis, Wintergreen, May 1997, p. 554.
[33] J.V. Crayford, D.H. Hutson, Pestic. Biochem. Physiol. 2 (1972) 295.
[34] S.A. Clay, V.C. Koskinen, Weed Sci. 38 (1990) 262.
[35] S. Fujiwara, S. Iwase, S. Honda, J. Chromatogr. 447 (1988) 133.