Anal Bioanal Chem (2005) 381: 879–883
DOI 10.1007/s00216-004-2971-9

ORIGINAL PAPER

M. Fátima Barroso · O. C. Nunes · M. Carmo Vaz
C. Delerue-Matos

Square-wave voltametric method for determination of molinate concentration in a biological process using a hanging mercury drop electrode

Received: 9 September 2004 / Revised: 8 November 2004 / Accepted: 17 November 2004 / Published online: 3 February 2005

Abstract A square-wave voltammetric (SWV) method using a hanging mercury drop electrode (HMDE) has been developed for determination of the herbicide molinate in a biodegradation process. The method is based on controlled adsorptive accumulation of molinate for 10 s at a potential of −0.8 V versus AgCl/Ag. An anodic peak, due to oxidation of the adsorbed pesticide, was observed in the cyclic voltammogram at ca. −0.320 V versus AgCl/Ag; a very small cathodic peak was also detected. The SWV calibration plot was established to be linear in the range 5.0×10^{-6} to 9.0×10^{-6} mol L^{-1}; this corresponded to a detection limit of 3.5×10^{-8} mol L^{-1}. This electroanalytical method was used to monitor the decrease of molinate concentration in river waters along a biodegradation process using a bacterial mixed culture. The results achieved with this voltammetric method were compared with those obtained by use of a chromatographic method (HPLC–UV) and no significant statistical differences were observed.

Keywords Hanging mercury drop electrode · Square-wave voltammetry · Molinate · Biodegradation

Introduction

Molinate (*S*-ethyl perhydroazepine-1-carbothioate) is a selective thiocarbamate herbicide used worldwide to control weeds in rice crops [1]. After being applied to the field it is mostly dissipated by evaporation (75–85%) and to a lesser extent by photolysis (5–10%) soil adsorption and biodegradation (5–10%) [2–5].

Besides physical phenomena, the concentration of molinate in the receiving environment can decrease as a result of chemical and biological processes involving oxidation of the sulfur, the azepine ring, and the ethyl moiety [4, 6–10]. Molinate and its degradation products have been detected in natural environments such as soil, rivers, lakes, underground streams, and supply waters, and in laboratory-scale decontamination systems [3, 11–18]. Despite of being considered a moderately toxic compound, molinate, and specially its oxidised derivatives, are toxic to warm-blooded animals and have been implicated in adverse reproductive and neurotoxic effects [19]. Consequently, it is important to develop strategies to treat molinate-polluted sites and avoid future contamination. Bioremediation seems to be an effective alternative to costly traditional physicochemical techniques used to treat contaminated soils and waters. Optimum bioremediation systems comprise microorganisms capable of readily mineralization of the target pollutant. In a previous study a mixed culture (DC) of five bacterial strains able to mineralise molinate was isolated and characterised [5]. Because mixed culture DC uses the herbicide as the only source of carbon and nitrogen without accumulation of dead end products, it has been used to implement biological decontamination processes [20].

Different analytical methods have been used to quantify molinate in different kinds of matrix. These include gas-chromatography (GC) with several types of detector, for example the nitrogen–phosphorus detector (NPD), the electron-capture detector (ECD) [21], the flame ionisation detector (FID) [5], and mass spectrometry (MS) [22], and liquid chromatography, HPLC, with UV detection [5, 23].

Considering that chromatographic techniques are expensive, labour-intensive, and involve sample-treatment stages that make laboratory analysis both time-consuming and environmentally more aggressive, because high quantities of contaminated effluent are

M. F. Barroso · M. C. Vaz · C. Delerue-Matos (✉)
REQUIMTE/Instituto Superior de Engenharia do Porto,
Rua Dr. Bernardino de Almeida 431, 4200-072 Porto, Portugal
E-mail: cmm@isep.ipp.pt
Tel.: +351-22-8340500
Fax: +351-22-8321159

O. C. Nunes
LEPAE-Departamento de Engenharia Química,
Faculdade de Engenharia da Universidade do Porto,
R. Dr. Roberto Frias, 4200-465 Porto, Portugal

produced, an alternative analytical method, using a glassy carbon electrode, has previously been proposed and applied successfully to quantification of molinate in phytopharmaceuticals [24]. The working concentration range was about 10^{-4} mol L^{-1}; for lower concentrations the voltammogram peak was not well defined and for higher concentrations strong adsorption on the electrode surface was observed, meaning that frequent cleaning of the electrode surface was necessary. This is an important drawback of voltammetry on solid electrodes. The limit of detection of molinate was 8.7×10^{-5} mol L^{-1}.

The aim of the current study was to study the behaviour of molinate at a hanging mercury drop electrode (HMDE) using voltammetric techniques, and the development of a method based on the electroactivity to monitor molinate concentration along a process of biodegradation by mixed culture DC.

Experimental

Equipment

All voltammetric measurements were performed using a computer controlled potentiostat/galvanostat Autolab PSTAT10 (EcoChemie, Netherlands), and a Metrohm 663 VA Stand containing a three-electrode cell (all Metrohm) with an HMDE, an Ag/AgCl/KCl 3.00 mol L^{-1} reference electrode, and a glassy carbon rod auxiliary electrode. The system was controlled by means of a general-purpose electrochemical system (GPES) software package, version 3.0, from EcoChemie.

All chromatographic measurements were performed by HPLC (Knauer) on a LiChrospher 5 μm RP-18 column (Merck) with UV detection (Knauer) at 210 nm. An 80:20 (*v*/*v*) methanol–water mixture was used as mobile phase at a flow rate of 0.8 mL min^{-1}. Cell-free supernatant or dilutions thereof were analysed directly by HPLC.

pH was measured with a Metrohm E-520 pH meter with combined glass electrode.

Reagents and solutions

Molinate (Pestanal grade, 99% purity) was obtained from Riedel–de Haen. All other reagents were Merck p.a. quality. Purified nitrogen 5 for voltammetric measurements was obtained from Linde, Sogás.

All aqueous solutions were prepared using purified water (conductivity <0.1 μS cm^{-1}) obtained from a Barnstead E-pure 4 system.

The supporting electrolyte in voltammetric determinations was culture medium B [5], used to grow mixed culture DC, composed of: phosphate buffer (27 mmol L^{-1}, pH 7.2), $CaCl_2.2H_2O$ (0.2 mmol L^{-1}), NaCl (7.56 mmol L^{-1}), $MgCl_2.6H_2O$ (0.81 mmol L^{-1}), $FeCl_2.4H_2O$ (5.19 μmol L^{-1}), HCl (1.3 μL, 25%), $ZnCl_2$ (0.5 μmol L^{-1}), $MnCl_2.4H_2O$ (0.5 μmol L^{-1}), H_3BO_3 (0.4 μmol L^{-1}), $CoCl_2.6H_2O$ (0.7 μmol L^{-1}), $CuCl_2.2H_2O$ (0.1 μmol L^{-1}), $NiCl_2.6H_2O$ (0.1 μmol L^{-1}), and $NaMoO_4.2H_2O$ (0.2 μmol L^{-1}).

The ionic strength of the supporting electrolyte was adjusted to 0.3 mol L^{-1} with KCl 3 mol L^{-1}. In the pH optimisation study the support electrolyte was acidified or made alkaline with HCl 1 mol L^{-1} or NaOH 1 mol L^{-1}, respectively.

Preparation of standards and samples

Molinate stock solutions (1.00×10^{-3} mol L^{-1}) were prepared with an exact weight of the pure pesticide and diluted up to 50.00 mL with medium B. Standards used for optimisation studies and for construction of calibration plots were prepared by dilution of this stock solution with medium B.

Culture conditions

Evaluation of the ability of mixed culture DC to purify real contaminated waters was performed using water from the river Pranto, in central Portugal, collected at a site of discharge of tail waters from different paddy rice fields situated in the valley of this river. The river water was spiked with molinate to furnish a final concentration of 1×10^{-3} mol L^{-1}. The culture was grown in a batch reactor with a 300-mL working volume, controlled temperature (30°C), and agitation (magnetic stirring, 130 rpm). Aerobic conditions were maintained by use of an air pump, at a flow rate of 0.15 vols gas per vol liquid min^{-1} (VVM). The reactor was inoculated (10%, *v*/*v*) with a preculture of mixed culture DC grown aerobically in medium B with molinate (1×10^{-3} mol L^{-1}), for 24 h at 30°C and 120 rpm. A non-inoculated control was operated simultaneously to control both abiotic losses and biodegradation of molinate by autochthonous microbiota. Dry weight of cells was estimated by spectrophotometric ($OD_{610\ nm}$) determination and interpolation of a calibration plot of optical density against dry weight of cells [5]. Molinate content was determined in the culture supernatant or dilutions thereof by square-wave voltammetry (SWV) and HPLC. For both methods, each molinate determination was performed in triplicate, independently.

Results and discussion

Cyclic voltammetry

The electrochemical behaviour of 8.0×10^{-5} mol L^{-1} molinate in medium B was studied at an HMDE by

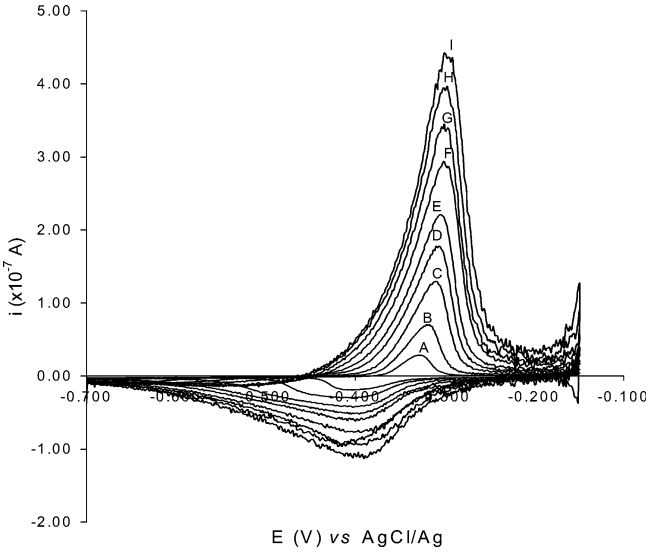

Fig. 1 Cyclic voltammograms of molinate solution at a concentration of 8.0×10^{-5} mol L^{-1} obtained with an HMDE: *A* 20 mV s^{-1}, *B* 50 mV s^{-1}, *C* 100 mV s^{-1}, *D* 150 mV s^{-1}, *E* 200 mV s^{-1}, *F* 300 mV s^{-1}, *G* 400 mV s^{-1}, *H* 500 mV s^{-1} and *I* 600 mV s^{-1}

cyclic voltammetry (CV). Figure 1 shows one oxidation peak at ca. −0.320 V versus AgCl/Ag in the anodic scan and a very short cathodic peak at the inverse scan for scan rates (v) between 20 mV s^{-1} and 600 mV s^{-1}, after accumulation at −0.800 V versus AgCl/Ag for 10 s. The short preconcentration time applied resulted in substantial enhancement of peak intensity compared with the signal obtained without deposition. Plots of current peak intensity (I_p) against $v^{1/2}$ were linear (R^2 = 0.998) and passed through the origin, which proved oxidation was diffusion-controlled.

pH study

After observing the electrochemical oxidation of molinate at the HMDE, a more sensitive and rapid technique, SWV, was used to assess the effect of pH on molinate peak shape and peak height (Fig. 2). Figure 2 shows that very little change was observed in the I_p for molinate electroactivity in the pH interval considered (from 1.9 to 11.0). For the optimum pH choice of pH 7.4 seemed the most appropriate considering the pH of the culture medium used for biodegradation of the pesticide or river water, where pH was always controlled. No effect on peak potential was observed for the pH interval studied.

Optimising experimental conditions

The experimental conditions in SWV are interrelated and have a combined effect on I_p. Hence, to establish the optimum conditions for determination of molinate, the influence of conditions such as frequency, f, deposition potential, P_d, deposition time, t_d, on the height peak of molinate was studied.

The frequency was varied from 20 Hz to 200 Hz. I_p increased with frequency until 150 Hz; above this value, however, a decrease in peak definition was also observed. Hence, this frequency was chosen for all subsequent measurements. The influence of deposition potential on the peak height was studied in the range −1.2 to −0.6 V versus AgCl/Ag. A maximum was observed at −0.8 V so this deposition potential was used in further analyses.

The effect of accumulation time (between 0 s and 40 s) of 5.0×10^{-6} mol L^{-1} molinate was investigated. The peak current increased linearly up to a maximum at 10 s and then remained constant indicating electrode saturation. All the analyses were carried out with 10 s deposition time.

With the optimised experimental conditions linearity studies were performed on peak current and molinate concentration.

When standard calibration graphs for molinate solution (Fig. 3) were plotted in the range 5.0×10^{-6} to 9.0×10^{-6} mol L^{-1} good linearity was obtained [I_p ($\times 10^{-7}$

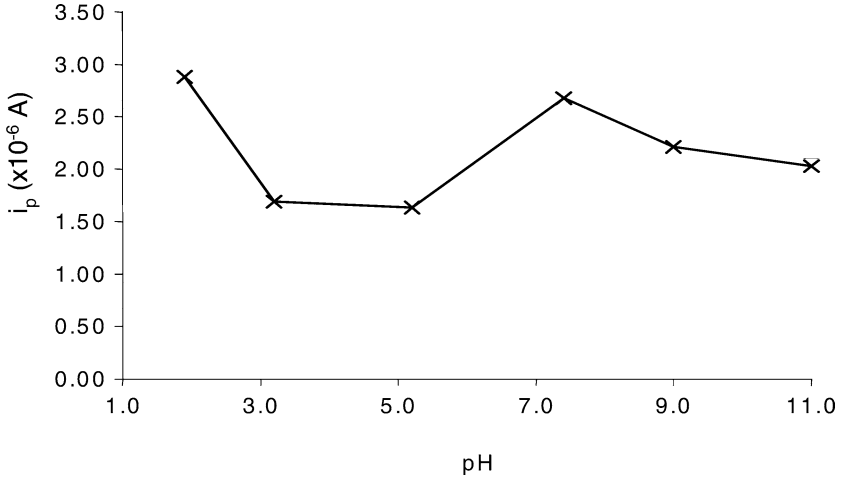

Fig. 2 Effect of pH on response values, I_p, for molinate solutions with a concentration of 2.2×10^{-5} mol L^{-1}, using the SWV technique

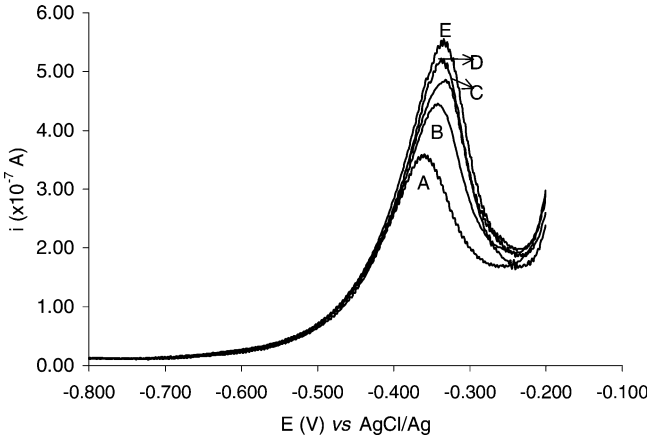

Fig. 3 Square-wave voltammograms of standard solution of molinate: *A* 5.0×10^{-6} mol L^{-1}, *B* 6.0×10^{-6} mol L^{-1}, *C* 7.0×10^{-6} mol L^{-1}, *D* 8×10^{-6} mol L^{-1}, *E* 9.0×10^{-6} mol L^{-1}

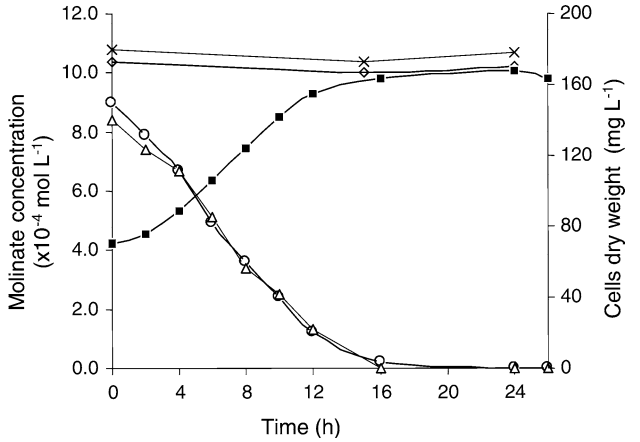

Fig. 4 Growth and molinate depletion by mixed culture DC in river water spiked with molinate. Growth curve (*filled squares*); molinate concentration in culture supernatant, quantified by HPLC (*open circles*) and by HMDE (*open triangles*); the concentration of molinate in the abiotic control was determined by HPLC (*crosses*) and by HMDE (*open diamonds*)

A) = $(6.9\times10^{-9} \pm 0.6\times10^{-9})$ A + $(4.9\times10^{-2} \pm 0.3\times10^{-2})$ concentration ($\times10^{-6}$ mol L^{-1})] with a correlation coefficient of 0.996. The limit of detection, calculated according to Miller [25, 26] was 3.5×10^{-8} mol L^{-1}.

Molinate quantification in a biodegradation process

The electrochemical method (SWV) developed was used to assess the decrease of molinate concentration in culture supernatant along a biodegradation process using mixed culture DC. The values obtained with this method were compared with those obtained by HPLC.

Mixed culture DC was described as able to mineralise free or adsorbed molinate when grown in synthetic culture medium [5, 20]. To evaluate whether this culture was able to degrade the herbicide in natural contaminated waters, biodegradation was conducted in water from a river located in a rice-producing area. Before being spiked with molinate the river water had a conductivity of 1,000 mS cm^{-1}, pH 7.4, and a total organic carbon content of 5.8 mg L^{-1}. Mixed culture DC was able to grow and to degrade the molinate present in the spiked river water without addition of any other nutrient (Fig. 4). The association between cell growth and molinate depletion was evidenced by the fact that the exponential growth phase was coincident with molinate consumption, and growth ceased when the molinate concentration was below the limit of detection of the analytical methods used (3.5×10^{-8} mol L^{-1}). Growth and herbicide depletion patterns observed for mixed culture DC grown in molinate-containing river water were similar to those obtained previously in synthetic medium [5, 20]. These results show that mixed culture DC can be used in future to decontaminate natural waters.

The suitability of this electroanalytical method for estimation of molinate in culture supernatant was assessed by analysing each sample in three independent analyses by the two different methods (SWV and HPLC). The mean values of molinate concentration and

Table 1 Variation with time of results from determination of molinate in culture supernatant, using HMDE and HPLC

Time (h)	Voltammetry ($\times10^{-4}$ mol L^{-1}; $n=3$)	HPLC ($\times10^{-4}$ mol L^{-1}; $n=3$)	RD (%)	Recovery (%)	
				HPLC	Voltammetry
0	8.4 ± 0.1	9.0 ± 0.1	−6.7	94	93
2	7.4 ± 0.1	7.9 ± 0.1	−6.3	–	–
4	6.7 ± 0.3	6.7 ± 0.2	0.0	–	–
6	5.1 ± 0.3	4.9 ± 0.1	+4.1	–	–
8	3.4 ± 0.1	3.6 ± 0.1	−5.6	–	–
10	2.5 ± 0.1	2.4 ± 0.2	+4.2	100	94
12	1.3 ± 0.1	1.2 ± 0.1	+ 8.3	–	–
24	ND	ND	–	–	–
26	ND	ND	–	106	94
C1	10.4 ± 0.1	10.8 ± 0.1	−3.7	106	96
C2	10.0 ± 0.1	10.4 ± 0.1	−4.0	–	–
C3	10.2 ± 0.2	10.7 ± 0.1	−4.7	–	–

ND, not defined; C1, abiotic control after 2 h; C2, abiotic control after 15 h; C3, abiotic control after 24 h

the standard deviations (below 0.3%) are presented in Table 1. Validation of the results was confirmed by comparison with the values obtained by HPLC determination, because the relative errors were always less than 7%. The recovery results, which were close to 100%, are also reported in Table 1. There is a good match between values obtained by the voltammetric method and by HPLC–UV. Study of the linearity of these techniques revealed a slope almost equal to unity and the intercept close to the origin ([HMDE]($\times 10^{-4}$ mol L^{-1}) = 5.64×10^{-6} mol L^{-1} + 9.54×10^{-1} [HPLC] ($\times 10^{-4}$ mol L^{-1}) R^2 = 0.999).

Similar results were obtained when molinate content was determined by both methods (SWV and HPLC) in mixed culture DC supernatant grown in synthetic medium B with molinate (1×10^{-3} mol L^{-1}). These results show that the developed voltammetric method can be used successfully to quantify molinate in biodegradation processes. Furthermore, adaption of this method to online monitoring might prove to be a valuable analytical tool for such biodegradation processes.

Conclusions

On the basis of the electrochemical behaviour of molinate at an HMDE, with potential detection of −0.320 V versus AgCl/Ag, an alternative method was developed for quantification of molinate in biological samples. The method was shown to a good alternative to the comparison method (HPLC), because it is simpler, faster, less expensive, does not involve sample preparation techniques, and differences between the results obtained by the two methods are not statistically significant. The alternative method produces smaller amounts of residual solutions compared with HPLC. The total volume per analysis by the voltammetric method is 10 mL given that all standard solutions are prepared in the electrochemical cell, whereas large volumes of mobile phases are needed for HPLC analysis. When comparing both methods for routine analysis, the voltammetric method also has an advantage considering the waste management issue.

Acknowledgements This work was partially financially supported by Fundação para a Ciência e a Tecnologia from Ministério da Ciência e do Ensino Superior, Portugal through FEDER funds (project POCTI/12193/1998).

References

1. Imai Y, Kuwatsuka S (1986) J Pesticide Sci 11:57–63
2. Rajagopal BS, Brahmaprakash GP, Reddy BR, Singh UD, Sethunathan N (1984) Residue Rev 93:1–199
3. Mabury SA, Cox JS, Crosby DG (1996) Rev Environ Contam Toxicol 147:71–117
4. Soderquist CJ, Bowers JB, Crosby DG (1977) J Agric Food Chem 25:940–945
5. Barreiros L, Nogales B, Manaia CM, Ferreira ACS, Pieper DH, Reis MA, Nunes O (2003) Environ Microbiol 5:944–953
6. Imai Y, Kuwatsuka S (1982) J Pesticide Sci 7:487–497
7. Thomas VM, Holt CL (1980) J Environ Sci Health B 15:475–484
8. Golovleva LA, Finkelstein ZI, Popovich NA, Skryabin GK (1981) Izv Akad Nauk SSSR Ser Biol 3:348–358
9. Jewell WT, Hess RA, Miller MG (1999) Drug Metab Dispos 27:842–847
10. Konstantinou IK, Zarkadis AK, Albanis TA (2001) J Environ Qual 30:121–130
11. Carrasco JM, Sabater C, Alonso JL, Gonzalez J, Botella S, Amoros I, Ibañez MJ, Boira H, Ferrer J (1992) Sci Total Environ 123(124):219–232
12. Ross LJ, Sava RJ (1986) J Environ Qual 15:220–224
13. Carrasco JM, Planta MJ (1987) Assoc Off Anal Chem 70:752–753
14. Imai Y, Kuwatsuka S (1988) J Pesticide Sci 13:247–252
15. Julli M, Krassoi FR (1995) Bull Environ Contam Toxicol 54:690–694
16. Cerejeira MJ, Pereira T, Silva-Fernandes A (1998) Chemosphere 37:2949–2955
17. Tsuda T, Kojima M, Harada H, Nakajima A, Aoki S (1998) Bull Environ Contam Toxicol 60:151–158
18. Viana P, Batista S, Pereira T, Silva E, Valério MJ, Silva A, Ferreira M, Silva-Fernandes AM (2003) Water Res 37:1055–1063
19. Cochran RC, Formoli TA, Pfeifer KF, Aldous CN (1997) Regul Toxicol Pharmacol 25:146–157
20. Silva M, Mendes A, Manaia CM, Fernandes A, Nunes OC (2004) Water Res 38:2677–2684
21. Ruiz MJ, Redondo MJ, Font G (1997) J Chromatogr A 776:348–354
22. Aguilar C, Borrull F, Marcé RM (1997) J Chromatogr A 771:221–231
23. Tjeerdema RS, Crosby DG (1988) Pesticide Biochem Physiol 31:24–35
24. Brett AMO, Garrido EM, Lima JLFC, Delerue-Matos C (1997) Portugaliæ Electrochimica Acta 15:315–319
25. Miller JN, Miller JC (2000) Statistics and chemometrics for analytical chemistry. Pearson Education, London
26. Mocak J, Bond AM, Mitchell S, Scollary G (1997) Pure Appl Chem 69:297–328