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DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR DETERMINING THE HERBICIDE MOLINATE WITH AND WITHOUT ALGINATE MICROPARTICLES

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Abstract

Molinate (S-ethyl-azepane-1-carbothioate) is a thiocarbamate herbicide used in rice cultivation for the control of grass weeds. Environmental contamination with molinate is of major concern due to the adverse effects described both for humans and animals.

Molinate hydrolase, a novel amidohydrolase previously characterized, is responsible for the initial breakdown of molinate, cleaving the thioester bond of molinate, releasing ethanethiol and azepane-1-carboxylate (ACA). Biotechnology is the key for sustainable farming. With advances in biotechnology, bioremediation has become one of the most rapidly developing fields of environmental restoration. Through the microencapsulation of molinate hydrolase, we are aiming to develop a bioremediation process for the effective molinate degradation in rice paddies.

The purpose of this work was to develop and validate an UV method to effectively quantify the substrate (molinate) in further assays with free and microencapsulated molinate hydrolase. The analytical method was validated and the main parameters, as limit of detection, linearity range, precision and accuracy were determined, and compared to those obtained by HPLC (regarding free enzyme kinetics). Both methods show to be linear ($r > 0.999$) over the concentration range of 0.005-0.150 mM molinate. The global uncertainty, estimated accordingly to the bottom-up approach used by Eurachem, was estimated for both methods.

The UV analytical method is effective and seems that it can be applied in future for the quantification of molinate breakdown by free and encapsulated molinate hydrolase.

Key words: microparticles, molinate, molinate hydrolase, uncertainty, UV

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1. Introduction

Biocatalysis, a crucial component of white or industrial biotechnology, relies on the broad catalytic activity of enzymes leading to the industrial (e.g., food, pharmaceutical) production of value-added compounds, or catalyzing the degradation of several environmental contaminants (e.g., pesticides, hydrocarbons) at low cost (Alcalde et al., 2006). This scientific area relies primarily on the search of microorganisms able to produce/degrade the target compound(s), characterization of the related/involved metabolic pathway(s), namely the identification, purification and characterization of the enzymes involved, and finally on the optimization of the process.

Thiocarbamates are a sub-group of the carbamate chemical family, which are used worldwide as herbicides (graminicides). Molinate (S-ethyl-azepane-1-carbothioate) is a thiocarbamate used in rice cultivation for the control of grass weeds, such as *Echinochloa spp*., *Glyceria spp*., and *Diplachne fusca* (Nunes et al., 2013). Environmental contamination with molinate is of major concern due

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to the adverse effects described both for humans and animals (Nunes et al., 2013). Molinate hydrolase, a novel amidohydrolase, previously characterized and heterologously expressed, is responsible for the initial breakdown of molinate, cleaving the thioester bond of molinate, releasing ethanethiol and azepane-1-carboxylate (ACA) (Fig. 1) (Duarte et al., 2011).

During the last few years, environmental awareness has been rising all over the world. European authorities aim at the development of a risk assessment system and a sustainable management plan for all types of water (surface, ground, drinking) that combines the requirements as defined by the Directive 414/91/EEC and those defined by the Water Framework Directive 2000/60/EC (EC, 2000; EEC, 1991). In order to address these new restrictions, the research interest was placed on establishing alternative, simple, low-cost technologies for the on-site treatment of wastewaters, natural and ground water, namely with biotechnology. With advances in biotechnology, bioremediation has become one of the most rapidly developing fields of environmental restoration (Dua et al., 2002). One of the techniques that can be used in the bioremediation of soils is the microencapsulation, which is a technique where liquid droplets, solid particles or gas compounds are entrapped in an encapsulating agent, forming small capsules with many useful properties. Microencapsulation can also provide a physical barrier between the core compound and the external environment (Estevinho et al., 2013a). In the case of the microencapsulation of enzymes the microcapsule can give extra protection to the enzyme against undesirable external conditions. Through the microencapsulation of molinate hydrolase, we are aiming to develop a bioremediation process for the effective molinate degradation in rice paddies.

Associated to this work, an adequate analytical method to control the applicability of this process needs to be developed. In industry, where large analytical series of similar samples are frequent and the search for quick, reproducible, precise, inexpensive and easy methods to analyze high amounts of samples is crucial, the UV methods represent an extremely adequate alternative. Nevertheless, literature still lacks information about the validation parameters and the uncertainty of the methodology accordingly to the most recent statistical techniques (Ellison and Williams, 2007; Estevinho et al., 2013b). Hence, the purpose of this work was to develop and validate an UV method to effectively quantify the substrate (molinate) in aqueous matrices, and aqueous matrices with alginate used in the preparation of microcapsules. The enzyme will be microencapsulated in alginate and the validated method that can be used to evaluate the enzyme's activity and also to test the influence of the biopolymer in the molinate analyses is here in presented. Alginate can also be used as a biopolymer model to test the influence of additional biopolymers in the solutions containing molinate. The analytical method was validated and the main parameters, as limit of detection, linearity range, precision and accuracy were determined, and compared to those obtained by HPLC. The study of the analytical methods focuses also in the determination of the global uncertainty associated to the results.

2. Material and methods

2.1. Reagents

Molinate (S-ethyl azepane-1-carbothioate) was obtained from Herbex, Produtos Químicos (Portugal). Sodium alginate (alginic acid, sodium salt) (180947-100 g) was obtained from Aldrich (USA). All other reagents were analytical grade and from commercial sources.

2.2. Preparation of standard and enzymatic solutions

Standard solutions were prepared from a stock solution of 2.0 M molinate in 50 mM phosphate buffer, pH 7.2. Two different calibration standards were prepared:

a) Calibration standards with 0.150, 0.100, 0.075, 0.050, 0.010 and 0.005 mM molinate prepared from the stock solution in phosphate buffer;

b) Calibration standards with 0.150, 0.100, 0.075, 0.050, 0.010 and 0.005 mM molinate prepared from the stock solution in 50 mM phosphate buffer, containing alginate microparticles (5 $mg.mL^{-1}$).

2.3. Alginate microparticles preparation

The alginate microparticles were prepared by a spray-drying technique. Spray-drying was performed using a spray-dryer BÜCHI B-290 advanced (Flawil, Switzerland) with a standard 0.5 mm nozzle.

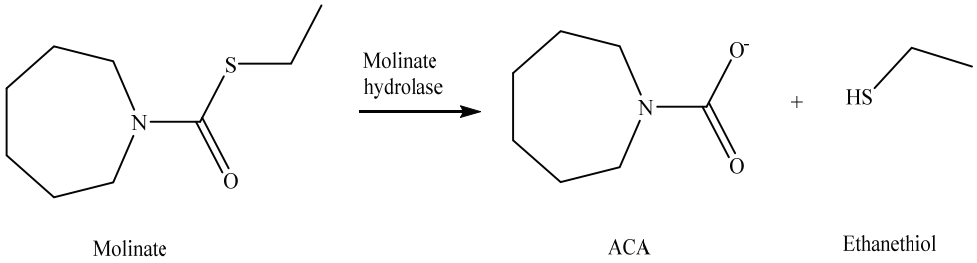

Fig. 1. Transformation of molinate by molinate hydrolase (Duarte et al., 2011)

The spray drying conditions, solution and air flow rates, air pressure and inlet temperature were set at 3 mL/min (10-13%), 35 $m^3.h^{-1}$ (90%), 6.0 bar and 115 ºC, respectively. The outlet temperature, a consequence of the other experimental conditions and of the solution properties, was around 45 ºC.

2.4. Analytical equipment

The equipment used was a T80/T80+UV-VIS spectrophotometer, (PG Instruments Ltd, England) and measurements were made at 230 nm wavelength, at room temperature.

2.5. Evaluation of the molinate concentration

The evaluation of the concentration of molinate was based on absorbance values, read in an UV-Visible spectrophotometer at 230 nm. Standard solutions were prepared with a concentration between 0.005 mM and 0.150 mM of molinate. The same procedure was used to prepare the standard solutions with molinate and alginate microparticles.

3. Results and discussion

In this work, an UV method was optimised and validated for the analysis of molinate in aqueous solutions with and without alginate microparticles. These analytical methods have been developed with the main objective of evaluating the degradation of molinate with the enzyme molinate hydrolase, in free and microencapsulated form. The method can be further used to evaluate the activity of the enzyme and also to test the influence of the biopolymer in the molinate analyses. Alginate can also be used as a biopolymer model to test the influence of additional biopolymers in solutions containing molinate.

The study of the analytical methods focuses not only the main characteristics of the method (linearity range, detection limit (LOD), accuracy and precision), but also on the determination of the global uncertainty associated to the results.

3.1. Validation of the UV method in aqueous matrices

The validation of this analytical methodology was performed for the analyses of molinate in a phosphate buffer. A calibration curve was obtained for six molinate standards with concentrations ranging from 0.005 to 0.150 mM. The values of the slope, intercept and correlation coefficient were 6.385, 0.029 and 0.995, respectively (Fig. 2).

The reproducibility of replicated analyses for each standard varied from 0.6 to 12.8%, when expressed as CV%. The LOD estimated from the calibration curve was 0.009 mM.

The intermediate precision of this method was evaluated taking into account the RSD of 6 analyses of each standard, on different days (Table 1). The CV% ranged from 1.2 to 14.1%. In general this value increased with the decrease of molinate concentration.

Accuracy was evaluated based on the recovery factor (%R), that was the ratio between the obtained concentration and the expected one. Accuracy is expressed by the percentage of recovery, for 6 experiments of each concentration level (Table 1).

Table 1. Intermediate precision and accuracy for molinate, at different concentration level, in phosphate solution

[Molinate] (mM)	*Intermediate precision (CV %)*	*Accuracy (Recovery %)*
0.150	2.9	98.2
0.100	4.0	103.0
0.075	1.2	106.1
0.050	6.4	102.1
0.010	14.1	70.5
0.005	8.6	29.5

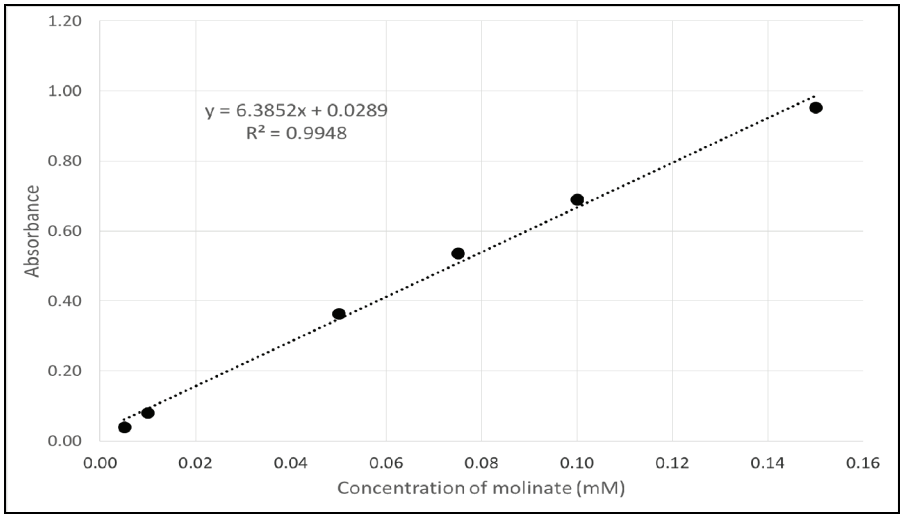

Fig. 2. Calibration curve for molinate in phosphate solution

The global uncertainty combines the contributions of all the sources of error linked to the analytical procedure. In this work, the bottom-up approach, adopted by the International Organisation for Standardisation (ISO), and also initially adapted for the analytical field by the EURACHEM/CITAC Guide (Ellison and Williams, 2007), was used to estimate the overall uncertainty by identifying, estimating and combining all the sources of uncertainty associated to the analytical results. An overestimation of the uncertainty is considered; this has the advantage of weighting the individual contributions and therefore allowing the detection of the most significant sources (Estevinho et al., 2008, 2009).

The global uncertainty (U) can be calculated from the following equation (Eq. 1):

$$U = \sqrt{U_1^2 + U_2^2 + U_3^2 + U_4^2} \quad (1)$$

where: U_1, U_2, U_3 and U_4 are the uncertainties associated to, respectively, standard preparation, calibration curve, precision, and accuracy.

The uncertainty associated to the standard preparation (U_1) was calculated for each standard according to Eq. (2), considering the relative error associated to each mass or volume measurement and the law of propagation of uncertainty. The standards were prepared by the method of the successive dilutions, so the lowest concentrations of the standards have incorporated all the errors associated to the preparation of the other standards.

$$U_1 = \sqrt{\left(\left(\frac{\Delta m_{balance}}{m_{molinate}}\right)^2 + \left(\frac{\Delta V_1}{V_1}\right)^2 + \left(\frac{\Delta V_2}{V_2}\right)^2 + \left(\frac{\Delta V_3}{V_3}\right)^2 + \ldots + \left(\frac{\Delta V_n}{V_n}\right)^2\right)} \quad (2)$$

where: $\Delta m_{balance}$ is the uncertainty associated to the mass measurement ($m_{molinate}$), ΔV_1 is the uncertainty associated to the preparation of the standard stock solution in a volumetric flask of 250 mL (V_1). ΔV_2 , ΔV_3 … ΔV_n are the uncertainty associated to the preparation of the standards (6 mL) and V_2, V_3, … V_n are the pipeted volumes to the preparation of the standard.

The uncertainty associated to the calibration curve (U_2) was calculated, for each standard, by the calibration curve, considering the following equations (Eqs. 3 and 4):

$$U_2 = \frac{\left(\frac{S_{y/x}}{b}\right) \times \sqrt{\left(\frac{1}{m}\right) + \left(\frac{1}{n}\right) + \left(\frac{(y_i - y_{av})^2}{b^2 \times \sum (x_i - x_{av})^2}\right)}}{x_0} \quad (3)$$

$$S_{y/x} = \sqrt{\frac{\sum (y_i - y_{ical})^2}{n-2}} \quad (4)$$

where $S_{\frac{y}{x}}$ represents the standard deviation of the linearization, b the slope of the regression curve, m the number of replicates performed for each xi value, n the number of standards used to build the calibration curve (n = 7), y_i the experimental value of y (absorbance), y_{ical} the value of y calculated by the regression curve for the concentration x_i, y_{av} the average of y_i values, x_i the concentration of standards (x) used in the calibration and x_{av} the average of x_i values.

The uncertainty associated to the precision (U_3) was estimated considering the precision of the measurement for each standard (Eq. 5). In the following formula, s represents the standard deviation of precision assays.

$$U_3 = \frac{s}{x \times \sqrt{n}} \quad (5)$$

The uncertainty associated to the accuracy (U_4) was calculated from Eq. 6, where $\boldsymbol{\eta}$ represents the recovery of the assays with spiked solutions at different levels:

$$U_4 = \frac{s(\eta)}{\eta_{average}} \quad (6)$$

In Fig. 3, the relative weight of each individual source of uncertainty for the molinate analysis is represented. The more important sources are the ones related with the calibration curve (U_2) and precision (U_3).

Fig. 4 presents the global uncertainty for the different concentration levels. The global uncertainty is close to 10% for concentrations levels between 0.07 and 0.15 mM. For lower concentrations these values increase to values up to 60% and 90% in the vicinity of the standard of 0.01 and 0.005 mM, respectively. In fact, the 0.005 mM standard presents a concentration smaller than the detection limit (0.009 mM) determined by the method, provoking this high value of uncertainty.

3.2. Validation of the UV method in aqueous matrices with alginate microparticles

This second method consisted in the analytical methodology for the analysis of molinate in a phosphate and alginate microparticles, testing by this way the influence of the alginate in the analysis of molinate. Again, the calibration curve was obtained for six molinate standards with concentrations ranging from 0.005 to 0.150 mM, prepared this time in a phosphate and alginate microparticles suspension. The values of the slope, intercept and correlation coefficient were 6.355 0.011 and 0.9996, respectively (Fig. 5).

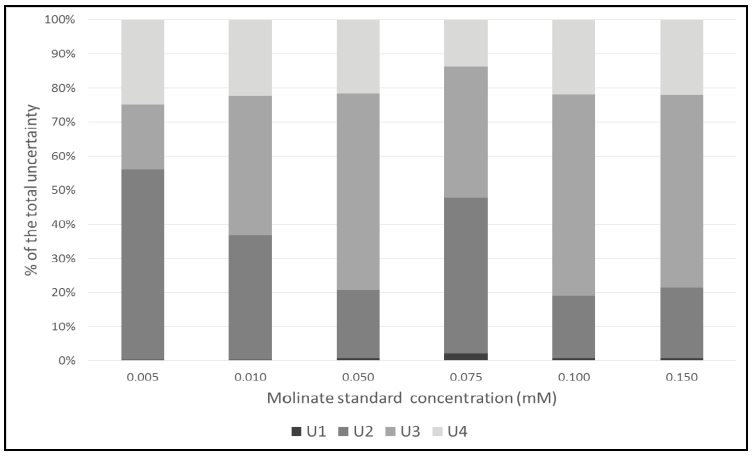

Fig. 3. The relative contribution of the uncertainty sources for different concentration levels of molinate

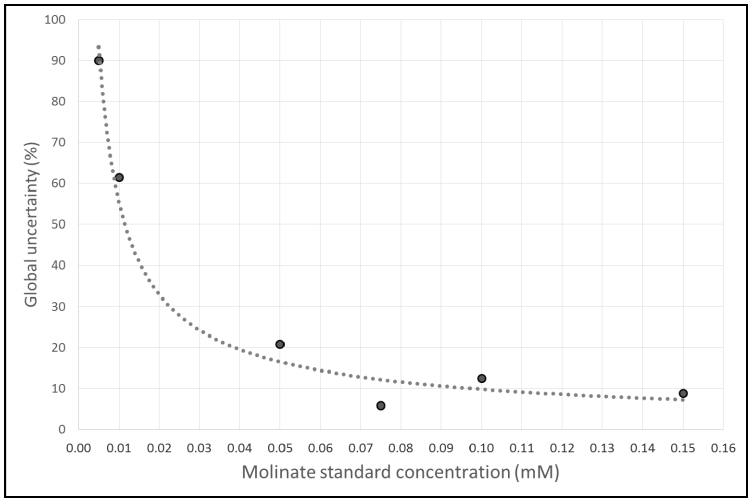

Fig. 4. Global uncertainty for molinate analysis by the UV method

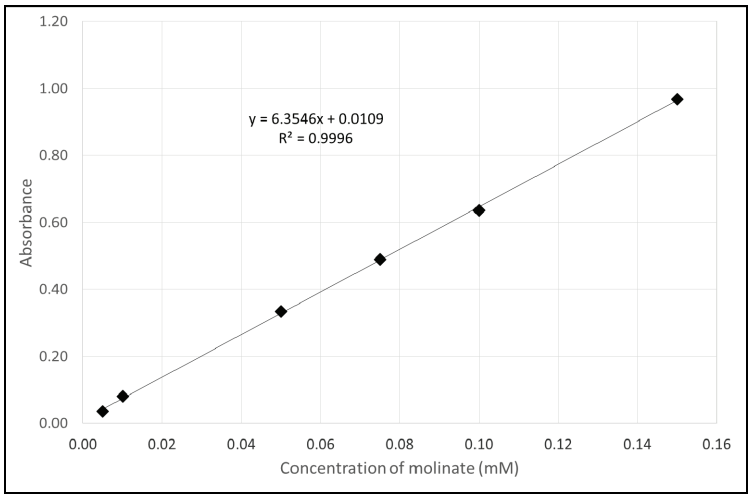

Fig. 5. Calibration curve for molinate in phosphate buffer and alginate microparticles suspension

The calibration curve is similar to the one determined without alginate in solution, this meaning that the interference of alginate is not significant.

The reproducibility of replicated analyses for each standard varied from 0.7 to 5.2%, when expressed as CV%.

The LOD estimated from the calibration curve was 0.002 mM, smaller than the one obtained for the other method. The results of the intermediate precision (CV%) and the accuracy, expressed on the recovery factor (%R of this method) are presented in Table 2.

Table 2. Intermediate precision and accuracy for molinate, at different concentration level, in phosphate and alginate microparticles suspension

[Molinate] (mM)	*Intermediate precision (CV %)*	*Accuracy (Recovery %)*
0.150	2.9	99.1
0.100	1.0	99.9
0.075	3.6	98.3
0.050	4.6	101.7
0.010	4.1	109.4
0.005	6.0	69.0

The global uncertainty and the contributions of the uncertainty sources for the different calibration levels in the molinate analysis were also determined. In Fig. 6 it is possible to observe the relative contribution of the uncertainty sources for different concentration levels of molinate measured in phosphate and alginate solution.

The relative contribution of these four sources is decisively dependent on the calibration levels. For this method the main sources are related to the precision (U_3) and the accuracy (U_4). However, for lower molinate concentrations, the uncertainty associated to the calibration curve (U_2) is the main responsible, almost 45%. For high molinate concentration levels the influences of precision (U_3) and accuracy (U_4) achieved a combined contribution around 90%.

Fig. 7 presents the variation of the global uncertainty, and it is observed that the global uncertainty is less than 10% for concentration levels between 0.05 and 0.15 mM. For lower concentrations the uncertainty increased in an exponential mode, the values increasing significantly to values higher than 25%.

Comparing the two UV methods, it can be observed that the alginate, the matrix that will be further used to microencapsulate the molinate hydrolase, do not influence significantly the precision and the accuracy of the method to determine molinate in aqueous solutions. The calibration curves are similar.

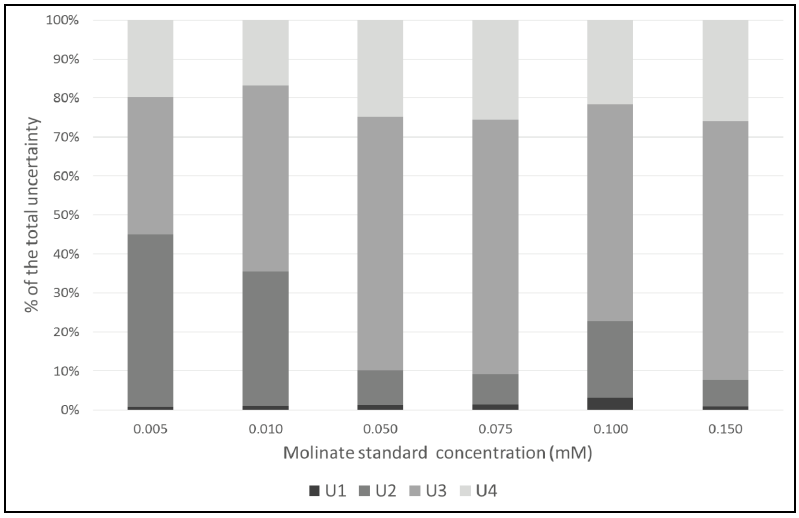

Fig. 6. The relative contribution of the uncertainty sources for different concentration levels of molinate measured in phosphate and alginate microparticles suspension

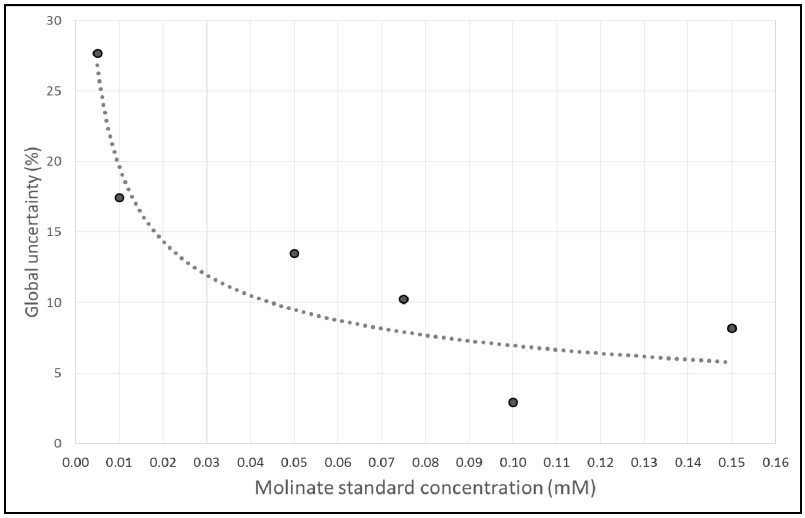

Fig. 7. Global uncertainty for molinate analysis by UV method measured in phosphate and alginate microparticles suspension

3.3. Molinate UV methods versus molinate HPLC method

The HPLC-UV method used by Barreiros et al. (2003) to quantify molinate was performed with a detection limit of 0.9 g/L (4.8 mM). In this work the HPLC-UV methodology was validated to analyse molinate, presenting a linear response between 0.005 and 0.2 mM (standards prepared in phosphate buffer), a correlation coefficient of 0.9999 and a detection limit of 0.001 mM. Global uncertainty associated with the results was around 8%. Some samples were analysed by the two methods (UV and HPLC) and the results obtained by the two methods are very similar. The HPLC method was developed for a more open range (0.005-0.200 mM), presenting a smaller detection limit (0.001 mM). However, the UV method has the advantage of avoiding the problems found in the chromatographic column, provoked by the mucoadhesive properties of alginate. This problem has already been described for other biopolymer, for the analysis of glucose in samples containing chitosan, by Estevinho et al. (2013b). On the other hand, when using the HPLC method, high costs are involved with acquisition and maintenance of the equipment, and acquisition of solvents and columns. The UV methods have smaller costs involved with the equipment.

The UV method is also more flexible and simple in the utilization, allows a large number of sample analyses in a small period of time, and presents less operating problems. The applicability of the UV method on the analysis of molinate in complex alginate samples proved to be a good option.

4. Conclusions

In this work, two UV methods to analyse molinate in phosphate buffer with and without alginate microparticles, were optimized and validated. It can be concluded that the alginate microparticles do not interfere in the molinate analysis, at least in the range of concentration used.

Both methods were shown to be linear ($r > 0.999$) over the concentration range of 0.005-0.150 mM molinate. The global uncertainty, estimated accordingly to the bottom-up approach used by Eurachem, was estimated for the two methods.

The UV analytical method is effective and can be applied for the quantification of molinate breakdown by free and encapsulated molinate hydrolase.

Acknowledgments

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