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Article

A Simple and Efficient Method for Derivatization of Glyphosate and AMPA Using 9-Fluorenylmethyl Chloroformate and Spectrophotometric Analysis

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Este trabalho apresenta a confirmação e otimização do método para derivatização de glifosato e ácido aminometilfosfônico (AMPA) utilizando cloroformiato de 9-fluorenilmetila (FMOC-Cl) avaliando-se os parâmetros: concentração de tampão borato e de FMOC-Cl, tempo de homogeneização e de reação, comprimento de onda e solvente de lavagem. A reação foi promovida com sucesso e o método é simples e de fácil execução pois utiliza cinco minutos de homogeneização e permite análises no ultravioleta imediatamente após o fim da reação.

This work presents the confirmation and optimization of the method for derivatization of glyphosate and aminomethylphosphonic acid (AMPA) using 9-fluorenylmethyl chloroformate (FMOC-Cl) evaluating the parameters: concentration of borate buffer and FMOC-Cl, homogenization time and of reaction, wavelength and solvent washing. The reaction was successfully promoted and the method is simple and easy to perform because it uses five minutes of homogenization and allows ultraviolet analysis immediately after the end of the reaction.

Keywords: FMOC-Cl, glyphosate, derivatization

Introduction

Herbicides are chemicals used in agricultural practices to remove competing plants in the first years of the development of a particular culture.[1] The use of these products has increased over the years due to intensive development of agriculture, making it necessary to know their behavior and effects on the environment.[1] Glyphosate (*N*-phosphonomethyl glycine, GLY) is a broad-spectrum herbicide used as the main active component of Roundup®, a weed control agent.[2] This herbicide is non-selective, systemic and post-emergent, representing 60% of the non-selective herbicides on the world market.[3] In several types of planting, it is usually pulverized, absorbed by plant leaves and new stalks and translocated to all parts through the route of photosynthesis products.[4] In its degradation, cleavage generates its main secondary metabolite, aminomethylphosphonic acid (AMPA).[5]

GLY and AMPA have been shown to be slightly toxic to mammals.[6] However, these highly soluble wastes are retained in the soil, transported to surface water and ground water and are then bioconcentrated in animals.[7] Aiming to monitor the application of this herbicide, some regulators have established maximum limits of these residues in the environment.[4] Thus, analytical methods such as liquid chromatography (LC) are being developed in order to analyze these compounds in environmental samples.[8]

In conventional detection systems, as with a ultraviolet (UV) detector, it is necessary that the molecule presents detectable characteristics, such as the presence of chromophore groups.[9] However, GLY and AMPA molecules do not have these groups,[10] thus hampering detection by spectrophotometric analysis in the UV range.[11] Thus, it is necessary to carry out a derivatization reaction to render this compound sensitive to analyses.

Derivatization is a chemical process that leads to new products with improved chromatographic properties.[12] Derivatization of GLY and AMPA may be used for analyses by gas chromatography (GC), UV or other methods.

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Analysis by LC is preferred due to the ionic character of the compounds.[9] Moreover, analyses by GC require a long period of time and considerable sample handling.[13] Derivatization for LC analyses may be done pre-column or post-column.[14] The most commonly used pre-column method is the reaction with the derivatizing agent 9-fluorenylmethyl chloroformate (FMOC-Cl). The reaction can be observed in Figure 1. In this process, substitution of a hydrogen atom in the amino group occurs in the GLY and AMPA molecules by aromatic rings containing alternating double bonds.[5]

Techniques conventionally used for the analysis of GLY and AMPA have used sophisticated methods with high cost, like LC coupled with UV detector,[2,9,11] fluorescence[5,8,15,16] and mass spectrometry.[17,18] Furthermore, using FMOC-Cl requires a long time for homogenization[16,17] and compound analysis after the reaction.[2,16] Thus, in this study, we sought to optimize a fast, easy and efficient technique for the derivatization of GLY and AMPA using FMOC-Cl and analysis by UV.

Experimental

Reagents

Standard GLY (99.2% m/m) and AMPA (99.0% m/m) stock solutions were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were prepared in deionized water at a concentration of 500 mg L^{-1} and stored at 4 °C. Working solutions were prepared from individual stock solutions at a concentration of 50 mg L^{-1} in the same solvent.

As solvents, acetonitrile (HPLC grade) obtained from Vetec (Rio de Janeiro, Brazil) was used. As the derivatizing agent, 9-fluorenylmethyl chloroformate (FMOC-Cl) (97% m/m) obtained from Sigma-Aldrich (St. Louis, MO, USA) was used. Sodium borate (99% m/m) was obtained from QM (São Paulo, Brazil).

Instruments

The absorption spectrum of the samples was obtained by analysis using a Cary 60 UV-Vis spectrophotometer (Agilent, Australia) with readings in the region of 190-340 nm.

Derivatization reaction

To ensure that the proposed methodology of derivatization generates reliable information, it was necessary to confirm that the process was occurring during the derivatization of the compounds under study. The following experiments were performed according to the following methodology: the reaction medium, whose composition is detailed in items (*i*)-(*iii*), was transferred to a round bottom flask (25 mL) and homogenized by vortexing for 30 minutes. Subsequently, it was washed with 7.50 mL of diethyl ether (twice) and 750 µL of the derivatized compound was diluted into 2.25 mL of borate buffer and analyzed on a UV-Vis spectrophotometer immediately after.

(*i*) Absorption of FMOC-Cl in the reaction medium without GLY: derivatization of the reaction medium was

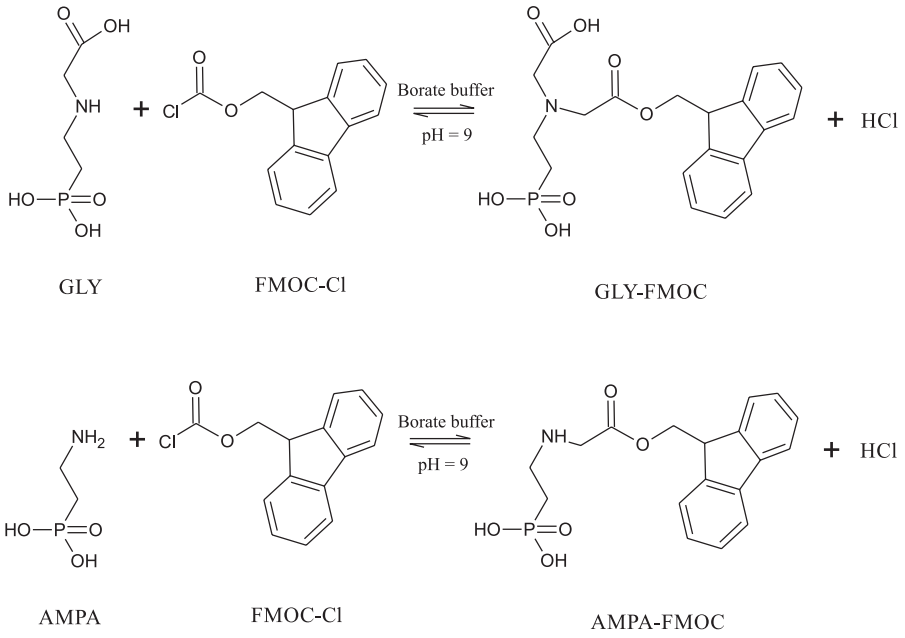

Figure 1. Derivatization reaction of GLY and AMPA using FMOC-Cl, showing the compounds formed after employing the pre-column method.[5]

carried out with 3.750 mL of deionized water, 1.875 mL of borate buffer and 3.750 mL of 500 mg L^{-1} FMOC-Cl in acetonitrile.

(*ii*) Absorption of GLY in the reaction medium without FMOC-Cl: the reaction medium was carried out with 3.750 mL of 500 mg L^{-1} GLY in deionized water, 1.875 mL of borate buffer and 3.750 mL of acetonitrile. The same procedure was used for AMPA.

(*iii*) Absorption of GLY in the reaction medium with FMOC-Cl: derivatization of the reaction medium was carried out with 3.750 mL of 500 mg L^{-1} GLY in deionized water, 1.875 mL of borate buffer and 3.750 mL of 500 mg L^{-1} FMOC-Cl in acetonitrile. The same procedure was used for AMPA.

In addition to these experiments, an FMOC-Cl solution (50 mg L^{-1}) prepared in acetonitrile was analyzed in the ultraviolet range to evaluate the FMOC-Cl absorption.

Optimization of derivatization

In the process of optimizing the derivatization of GLY and AMPA using FMOC-Cl, variables that displayed significant effects were assessed in order to obtain a greater percentage of the derivatized product, faster sample preparation and lower cost. Some factors were evaluated and are shown in Table 1.

Table 1. Parameters assessed in the univariate optimization of the derivatization of GLY and AMPA

Variable	Level
Buffer concentration / (mmol L^{-1})	2, 20 and 200
FMOC-Cl concentration / (mg L^{-1})	200 and 500
Homogenization time / min	5, 10, 20 and 30
Reaction time after reaction / h	0, 4 and 8
Wavelength / nm	210 and 260
Washing solvent	Diethyl ether and ethyl acetate

In a 25 mL round bottom flask, 3.750 mL of the standard solution of 500 mg L^{-1} GLY, 1.875 mL of sodium borate and 3.750 mL of FMOC-Cl in acetonitrile were added in this order and subjected to homogenization by vortex. To remove the excess derivatizing agent (FMOC-Cl), two successive washes were performed using 7.5 mL of nonpolar organic solvent (Table 1). After this step, 750 µL of the sample was diluted in 2.250 mL of sodium borate buffer and analyzed by a UV-Vis spectrophotometer. The best conditions were determined according to the absorbance found in the UV-Vis spectrum.

Results and Discussion

Efficient derivatization of GLY and AMPA is required due to the molecular characteristics of these compounds that reduce their sensitivity to analytical techniques such as gas chromatography and analysis by UV-Vis spectroscopy.[6] The derivatization reaction using FMOC-Cl is an alternative due to the high sensitivity of this compound in the ultraviolet region. During this study, the efficiency of derivatization was verified and the parameters used to increase the sensitivity of the results were evaluated as follows:

Spectral behavior in the derivatization

To confirm the effectiveness of the derivatization reaction, some procedures were performed and the results are reported in Figure 2.

When evaluating the results, it was found that FMOC-Cl in the pure solvent had a sharp absorption band in the UV-Vis range (Figure 2a). It was observed that the 50 mg L^{-1} solution provided a spectrum containing two regions of high absorbance (210 and 260 nm). With the study of the absorption of FMOC-Cl in the reaction medium without the analytes (GLY and AMPA), the same profile was observed as for FMOC-Cl in pure solvent, although the maximum absorbance (at 210 nm) was about 0.1 (Figure 2b). This significant decrease in the absorbance of FMOC-Cl was due to the removal of excess compound after washing with an organic solvent (diethyl ether).

The glyphosate molecule has C=O and P=O bonds that may cause absorption in the ultraviolet range from 198 to 202 nm. Thus, absorbance for GLY (ca. 0.1) was observed when the absorption in the reaction medium was analyzed (Figure 2c). These results indicate that the other components of the reaction medium do not influence the absorption and confirm the necessity of derivatizing GLY to increase the sensitivity of analytical techniques such as UV-Vis spectroscopy. The same result was obtained for AMPA in the reaction medium (Figure 2d).

For the analysis of GLY and AMPA, the reaction medium containing FMOC-Cl provided an absorption spectrum (Figures 2e and 2f) with a similar profile to that obtained for FMOC-Cl in the pure solvent (Figure 2a). This analysis confirms that FMOC-Cl reacts with GLY and AMPA molecules, resulting in an amphipathic compound (with the polar part derived from glyphosate or AMPA and nonpolar part derived from FMOC-Cl). Thus, during washing with the organic solvent only the excess of FMOC-Cl (which did not react GLY and AMPA molecules) was removed and the FMOC-GLY

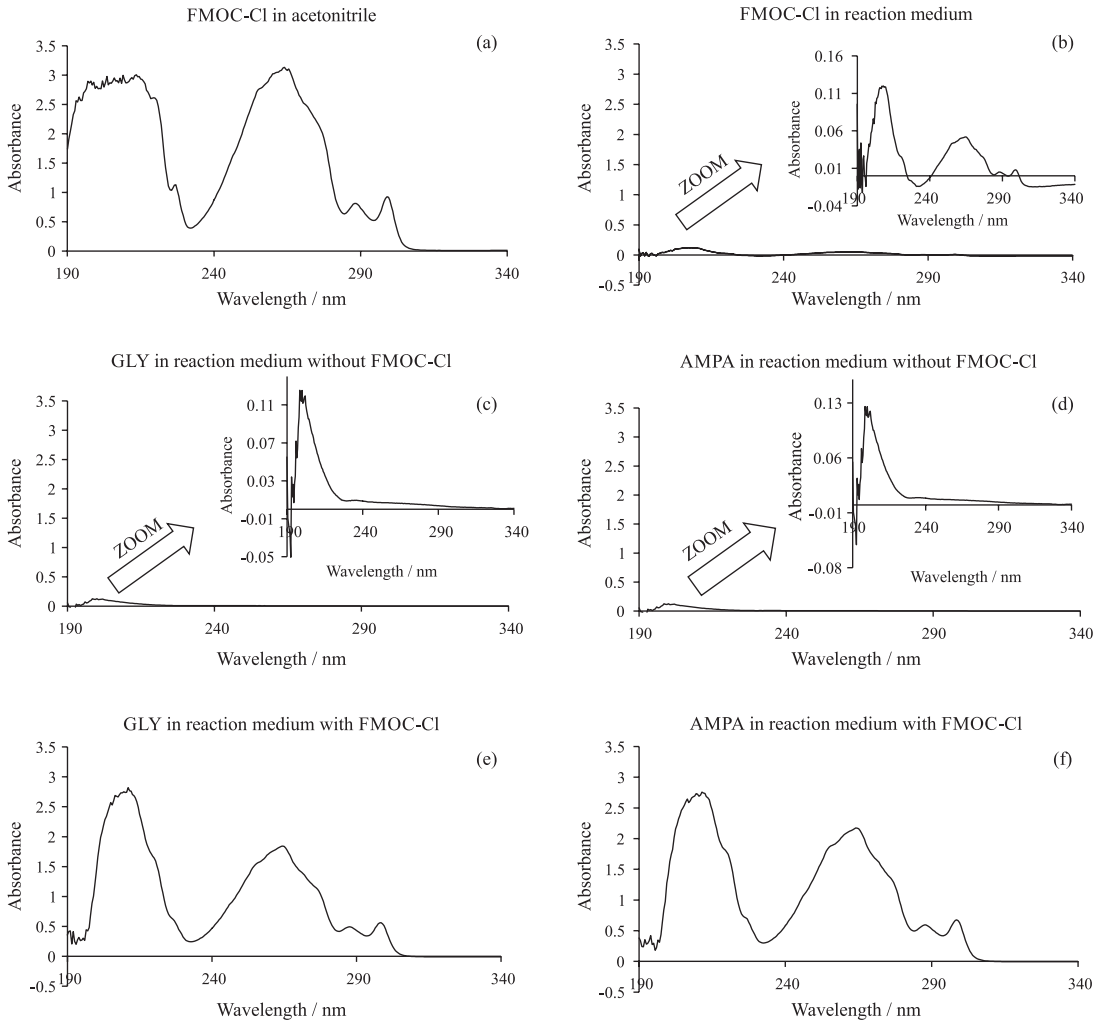

Figure 2. The ultraviolet absorption spectrum of: (a) 50 mg L^{-1} FMOC-Cl in acetonitrile; (b) FMOC-Cl in the reaction medium; (c) and (d) GLY and AMPA in the reaction medium, respectively; (e) and (f) GLY and AMPA in the reaction medium containing FMOC-Cl, respectively.

and FMOC-AMPA compounds were retained in the medium, generating the absorption profile characteristic of FMOC-Cl.

Optimization of derivatization

Effect of the borate buffer concentration

To verify if the concentration of borate in the reaction medium directly interferes with the derivatization reaction, 2, 20 and 200 mmol L^{-1} borate buffer were evaluated. The results are shown in Figure 3.

It was observed that at lower concentrations (2 mmol L^{-1}), the derivatization reaction did not occur (Figure 3). However, the instrument response was increased with an increasing concentration of borate. The concentration of 20 mmol L^{-1} showed maximum absorption (260 nm) of ca. 0.5, while absorbance with 200 mmol L^{-1} was ca. 3. It was found that increasing the buffer concentration in the medium promotes the reactivity of the amine function

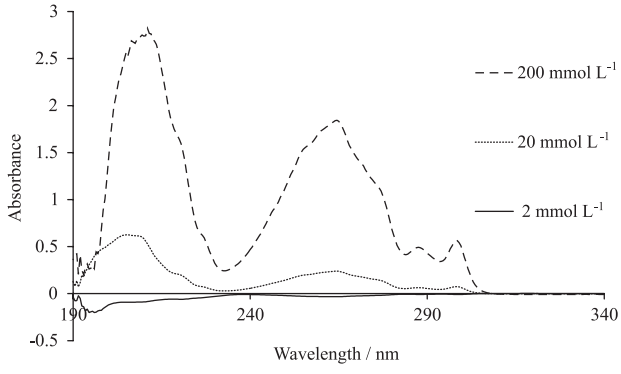

Figure 3. Ultraviolet spectrum of a 10 mg L^{-1} GLY solution derivatized with FMOC-Cl utilizing sodium borate buffer at 2, 20 and 200 mmol L^{-1}. The spectrophotometric analysis performed in scan mode demonstrated that there was higher absorption of the compounds at 210 and 260 nm.

and stabilizes the solubility of the derivatizing reagent in acetonitrile,[14] favoring the process of derivatization. Thus, sodium borate solution at a concentration of 200 mmol L^{-1} was selected for subsequent tests.

Effect of the FMOC-Cl concentration

The stoichiometric ratio between the analyte and the derivatizing reagent affects derivatized product formation.[19] Previous studies indicated that a 1:1 ratio was ineffective for the reaction.[19] Thus, this study used 200 and 500 mg L^{-1} FMOC-Cl, representing the proportions of 1:2 and 1:5 for the analyte and FMOC-Cl, respectively. The spectral behavior of these proportions can be seen in Figure 4, which also shows the increase in absorption with increasing the ratio of GLY:FMOC-Cl from 1:2 to 1:5.

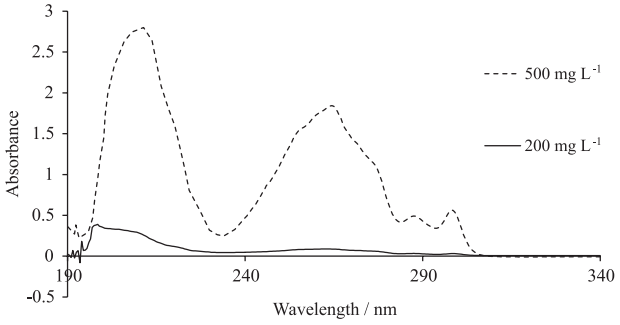

Figure 4. Ultraviolet spectrum of the solution of 10 mg L^{-1} GLY derivatized with FMOC-Cl at 200 and 500 mg L^{-1}.

From these results, it was found that a higher proportion of derivatizing agent (1:5) increased the absorption maximum (210 nm) by 1048% (Figure 4). At higher concentrations, the excess derivatizing agent, which becomes FMOC-OH, does not interfere in the analysis, because the excess is removed from the system during the washing step with organic solvents.[19] At the lower concentration of the derivatizing reagent, analyte detection becomes more difficult[5] (Figure 4) since the lowest concentration of molecules decreases the probability of reaction. Thus, the concentration of 500 mg L^{-1} was found to be more efficient in this work.

Effect of the homogenization time

Homogenization provides greater interaction of the analyte with the derivatizing reagent. However, prolonged homogenization lengthens the duration of the procedure. In this study, times of 5, 10, 20 and 30 minutes were studied, as shown in Figure 5a.

Further homogenization time did not increase the derivatized product, GLY-FMOC (Figure 5a). Maximum absorption (0.7) was obtained for the derivatized product with 5 min of mixing (Figure 5a). Thus, 5 min was selected to ensure a fast and efficient method.

Effect of the reaction time

The reaction time is definitive to ensure derivatization because this time period ensures the replacement of chlorine

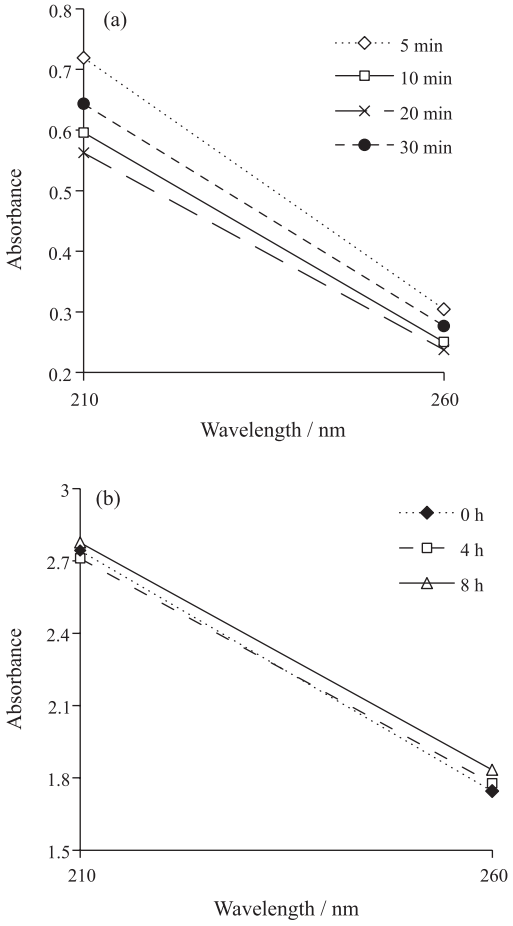

Figure 5. Ultraviolet spectrum of a solution of 10 mg L^{-1} GLY derivatized with FMOC-Cl with different homogenization times (a) and different reaction times (b).

in the structure of FMOC-Cl by the glyphosate molecule. However, studies in the literature indicate reaction times of 4-24 h, so there is no standard.[2,15] Thus, reaction times of 0, 4 and 8 hours were evaluated, and the results are shown in Figure 5b.

Spectrophotometric analysis immediately after the reaction (0 min), as well as 4 and 8 h after process showed that there was no significant variation in the absorbance obtained for different reaction times (Figure 5b). Therefore, it was concluded that the reaction is stopped immediately after homogenization and thus can be analyzed immediately after the procedure is performed.

Effect of the washing solvent

A decrease in the excess of FMOC-Cl in the sample was ensured with two successive washes with an organic solvent. Diethyl ether and ethyl acetate were used. Figure 6 shows the results.

According to the solvent present in the medium, variations in position, intensity and shape of the absorption band of the analyte due to solute-solvent interaction may

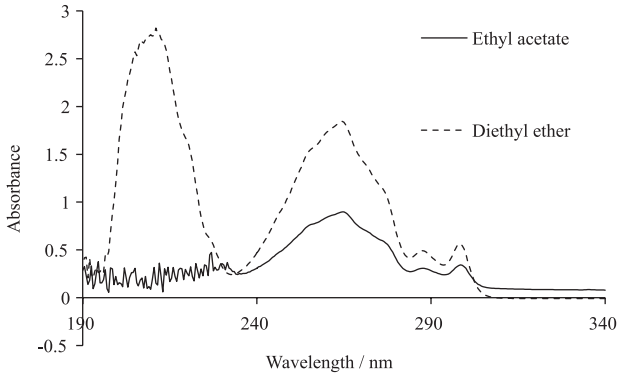

Figure 6. Ultraviolet spectrum of a solution of 10 mg L^{-1} GLY derivatized with FMOC-Cl and submitted to washing with ethyl acetate and diethyl ether.

occur.[20] In Figure 6, the UV analysis indicated that when using diethyl ether, the spectrum showed two regions of high absorbance (210 and 260 nm), while the ethyl acetate spectrum showed only one signal of high absorbance (260 nm). The signals at the same wavelength differ in absorption (Figure 6).

A similar result was obtained by Jamison *et al.*,[21] who showed that the increase of polarity of the solvent resulted in a decrease in the absorption due to the disappearance of the $n \rightarrow \pi^*$ transition bands. Thus, as it was observed that the resulting compound showed higher absorption after washing with diethyl ether, this was selected as the washing solvent in this study.

Conclusions

In this study, a derivatization technique was successfully optimized for the analysis of GLY and AMPA by ultraviolet spectrophotometry. The optimum conditions were homogenization by vortexing (5 min) using borate buffer solution (200 mmol L^{-1}) and FMOC-Cl (500 mg L^{-1}). The reaction was stopped immediately after washing with diethyl ether and the ideal wavelength for the analysis was 260 nm.

The confirmation of derivatization showed satisfactory results and showed that the reaction was in fact promoted.

The optimization of the derivatization using FMOC-Cl resulted in a simple method that was fast and efficient. The conditions were optimized and confirmed by UV-Vis spectrophotometry.

Acknowledgments

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