# GLYPHOSATE DETERMINATION IN NATURAL WATERS BY FLUORESCENCE SPECTROSCOPY AND SECOND-ORDER MULTIVARIATE CALIBRATION

*MANUEL A. BRAVO* *, *VERÓNICA ESCOBAR, SONNIA PARRA V., AND LUIS F. AGUILAR*

*Laboratorio de Química Analítica y Ambiental, Instituto de Química, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Avenida Brasil 2950, Valparaíso, Chile.*

**ABSTRACT**

In this work, 9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl) and 4-chloro-7-nitrobenzofurazane (NBD-Cl) were evaluated for glyphosate (Gly) determination by fluorescence and second-order multivariate calibration. Additionally, the analytical performance of the UPLS-RBL and MCR-ALS algorithms was compared based on figures of merit and prediction errors. The best results were obtained using NBD-Cl and UPLS-RBL, with a limit of detection (LOD) of 0.3-4.4 µg $L^{-1}$ and a prediction error (REP) of less than 2%. The FMOC-Cl showed similar figures of merit but inadequate fit in the presence of unexpected interferences. The proposed method was applied to analyze fortified well water, lagoon water, river water, and tap water. Because the matrix effect was significant, the analysis was performed with calibration in the matrix, yielding similar figures of merit and prediction errors to those of the validation set. These results evidenced the analytical potential of the proposed method for monitoring glyphosate in the hydrological cycle.

**Keywords:** *Glyphosate; Second-order Multivariate Calibration; Fluorescence spectroscopy; Water samples*.

## 1. INTRODUCTION

The N-(phosphonomethyl)glycine, commonly named Glyphosate (Gly), is a non-specific and broad-spectrum herbicide widely used in agriculture and horticulture for control weeds that affect cultivated crops [1, 2]. After application, this compound can be metabolized for microorganisms to aminomethylphosphonic acid (AMPA), absorbed by the leaves or translocated to plants by the roots and rhizomes through the phloem [3]. In soils, Gly is considered a non-mobile herbicide, being well-retained by clay particles, organic matter and iron hydroxides[4]. However, recent studies suggest that this compound is susceptible to leaching from soils towards aquatic environments, affecting the environment and producing toxic effect over non-target organisms [5-7]. Besides, the presence of Gly in food, such as cereals, fruits and vegetables suggest the exposition risk of human population to this compound [8, 9]. The International Agency for Research on Cancer (IARC) has been classified to Glyphosate as "probably carcinogenic to humans" (Group 2A) and registered strong evidence of genotoxicity, for "pure" glyphosate and for glyphosate formulations [2]. Some studies discard the relation between glyphosate exposition and cancer [10]. However, increasing evidence shows that glyphosate and glyphosate-based herbicides exhibit cytotoxic and genotoxic effects, increase oxidative stress, disrupt the estrogen pathway, impair some cerebral functions, between other effects [11, 12]. In any case, the control of Gly in environmental matrices, such as natural waters, appears mandatory to assess the human risk exposition to high concentrations of this herbicide.

Several analytical methods have been proposed for the determination of glyphosate in natural water. Commonly, chromatographic separation based on liquid chromatography (HPLC) coupled with mass spectrometer (MS) is used [13, 14]. However, strong matrix effects evidenced during complex sample analysis can reduce dramatically the sensitivity of mass spectrometer. Alternatively, fluorescence detection (FLD) has been used after a pre- or postcolumn derivatization procedure because glyphosate does not present native fluorescence [15, 16]. Different reagents, such as 9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl), 4-chloro-7-nitrobenzofurazane (NBD-Cl) or o-phthdialdehyde (OPA) have been proposed to obtain a fluorescent response [17, 18]. In general, these methods present high selectivity and sensitivity, and it has been applied satisfactorily for different environmental and food matrices. However, these are time consuming, requires high volume of organic solvents and it produces no-inert wastes. In addition, fluorescence assays were developed for the detection of glyphosate content based on carbon dots, quantum dots, paper-based, and aptamer-based with an interesting analytical potential [19]. Nevertheless, these approaches have showed limited applicability and selectivity deficiencies during complex sample analysis requiring complicated sample treatment steps[20].

The application of luminescent techniques, such as fluorescence spectroscopy appears as an alternative to quantification of organic pollutants due its high sensitivity and selectivity. However, the presence of unexpected interferences in complex samples makes non-viable the application of univariate quantification strategy and more complex data treatment are necessary. The second-order multivariate calibration has been proposed for this purpose, where three-way data is modelled by using some algorithms such as Parallel Factor analysis (PARAFAC) [21], Unfolded Partial Least Squares coupled to Residual Bilinearization (U-PLS/RBL)[22, 23] and Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS)[24]. These methods allows the determination of the compounds of interest, in a sample with interferences not included in the calibration set, property named second-order advantage [25-27]. These strategies have been used successfully for the determination of various contaminants such as pesticides [28], pharmaceuticals [29] and organic pollutants in several environmental matrices such as water, food, soil or sediment samples [30, 31].

In this work, a novel analytical method based on fluorescence spectroscopy coupled to second-order multivariate calibration for the determination of glyphosate in natural waters is proposed. In the literature, only two report based multivariate calibration to quantify glyphosate in water samples by using fluorescence spectroscopy has been proposed [32, 33]. However, in both studies only NBD-Cl was evaluated as fluorogenic agent, the performance of different chemometric algorithms was not compared and the study of interferences or the aqueous sample matrices evaluated was scarce or limited. In this study, the systematic and critical evaluation of fluorescence spectroscopy coupled to second-order multivariate calibration approach for glyphosate determination in natural waters. Firstly, different fluorogenic labelling reagents was evaluated in optimal conditions. Besides, the analytical performance of different algorithms was evaluated for the determination of Glyphosate in presence of unmodelled interferences and in spiked real water samples. In the best of our knowledge, no similar studies have been reported for glyphosate determination in natural waters.

## 2. THEORY

### *2.1. UPLS-RBL*

The U-PLS method, is a variant of classical Partial Least Squares (PLS) proposed for second-order data modelling, unfolding the data into vectors before two-way PLS calibration [34, 35]. If the calibration were exact, the regression

*Corresponding author email: manuel.bravo@pucv.cl

coefficients **v** could be employed to estimate the analyte concentrations $y_u$ in an unknown sample using the eq (1).

$$y_u = \mathbf{t}_u^T \mathbf{v} \quad (1)$$

where $\mathbf{t}_u$ (size A × 1) is the test sample score, obtained by projection of the data for the test sample $\mathbf{X}_u$ (size J×K, J and K are the number of instrumental channels in each data mode) onto the space of the *A* latent factors, commonly estimated by cross-validation. However, if unexpected interferents are presents in the sample, this mathematical solution is not exact, and the estimated concentration will be biased. In that case, U-PLS can be coupled to residual bilinearization procedure (RBL) to reach the second-order advantage. In this way, the matrix of sample containing unexpected interferences, $\mathbf{X}_u$, is vectorized to form vec($\mathbf{X}_u$) (JK × 1) and modelled in accordance with eq 2. .

$$\text{vec}(\mathbf{X}_u) = \mathbf{P}\mathbf{t}_u + \left[\mathbf{B}_{unx}\mathbf{G}_{unx}(\mathbf{C}_{unx})^T\right] + \mathbf{e}_{RBL} \quad (2)$$

where $\mathbf{t}_u$ (size A × 1) is the test sample score, **P** is the matrix of loadings of the UPLS-model (eq. 1), $\mathbf{e}_{RBL}$ is the residual error RBL term and $\mathbf{B}_{unx}$, $\mathbf{G}_{unx}$, and $\mathbf{C}_{unx}$ are provided by singular value decomposition based on $\mathbf{N}_{unx}$ unexpected components of the residual matrix obtained from eq(1). During the RBL procedure, loadings **P** in eq(2) in keeping constant at the calibration values and $\mathbf{t}_u$ is varied in order to minimize the norm of $\mathbf{e}_{RBL}$[36].

## 3. EXPERIMENTAL

### *3.1 Reagent and solutions*

High quality water (18 MΩ) obtained from a Barnstead Easypure II (Thermo, Dubuque, MA USA) was used to prepare the solutions.

Glyphosate PESTANAL® (99.7%), 9-fluorenylmethylchloroformate (FMOC-Cl) (97%) and 4-chloro-7-nitrobenzofurazane (NBD-Cl) (98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). For validation, (Aminomethyl) phosphonic acid (AMPA) (99%) and Cabofuran PESTANAL® (99.9%) were obtained from Sigma-Aldrich (St. Louis. MO, USA).

Besides, analytical purity grade Sodium borate ($Na_2B_4O_7 \cdot 10H_2O$, ≥ 99.5 %) and HCl (37% w/v) were obtained from Merck (Darmstadt, Germany). Solvents, such as Acetonitrile and methanol HPLC grade were purchased from Merck (Darmstadt, Germany).

Stock solutions of glyphosate (130 mg $L^{-1}$) were prepared weekly in deionized water. Besides, solutions FMOC-Cl (1.6 x$10^{-2}$ M) and NBD-Cl (3.8 x $10^{-3}$ M) were prepared daily in acetonitrile and methanol, respectively. All solutions were stored in amber vials at 4 ºC in darkness until analysis. Borate buffer is prepared to 0.2 M in deionized water, adjusting to pH 9.2 with HCl (0.1 M) or NaOH (0.1 M) solutions.

### *3.2 Instrumentation and materials*

A fast-scanning luminescence spectrometer model Cary-Eclipse (Agilent, Santa Clara, USA) equipped with a xenon flash lamp was used to obtain excitation–emission fluorescent matrices (EEFM) using a 3.00 ml fluorescence quartz cuvette (Starna cells, CA, USA). The EEFMs for NBD-Cl method were recorded at $\lambda_{exc}$ of 420-520 nm every 5 nm and in the $\lambda_{em}$ range of 490-650 nm every 2 nm, while for FMOC-Cl were recorded in the $\lambda_{exc}$ ranges of 200–310 nm every 5 nm and $\lambda_{em}$ of 280-400 nm every 2 nm. The excitation and emission slit widths were adjusted to 5.0 nm.

The EEFMs were saved in ASCII format and transferred to a portable computer for subsequent manipulation. The multivariate analysis was implemented in MATLAB R2012a and using graphical interface MVC2 [37]. This interface contains the matlab code for MCR-ALS y UPLS-RBL algorithms, figures of merits of calibration models based on uncertainty propagation [38]. The Rayleigh and Raman scatter signals from EEFMs were corrected with previously reported routines [39] written in MATLAB before analysis of calibration and validation set.

For FMOC-Cl method, the excess of free fluorophore was eliminated before fluorescence measurements using a 3M Empore™ C18 Extraction Disk (St. Paul, MN, USA) supported to a KS13 stainless steel syringe filter holder purchased from Advantec MFS (Dublin, CA). This method was adapted from previous work focused in glyphosate analysis using FMOC-Cl derivatization and chromatographic separation[40].

### *3.3* **Calibration and validation set samples.**

For calibration, ten standard solutions of glyphosate were prepared with concentrations between 0 and 150 µg $L^{-1}$. For this, adequate volumes of glyphosate solution were added into vial flask together with 300 µL of sodium borate buffer (0.2 M, pH: 9.2) and 2.0 mL of NBD-Cl solution. Subsequently, these solutions were heated at 90° C for 5 minutes, and then 45 µL of concentrated HCl were added. Finally, the volume was completed to 3.0 mL with methanol, and the EEFM was registered and analyzed according to the procedure described in section 2.2.

For calibration with FMOC-Cl, a modified procedure based on previous work was used [40]. Briefly, adequate volumes of the glyphosate standard were added with 120 µL of sodium borate buffer (0.2 M, pH: 9.5), 400 µL of acetonitrile and 60 µL of FMOC-Cl solutions. After 30 minutes, this solution was stirred in a vortex and 120 µL of borate buffer was added, and the final volume was adjusted to 5.0 mL with deionized water. Subsequently, 550 µL of each solution was passed through a C18 membrane, previously conditioned with 1.0 mL of methanol and 1.0 mL of deionized water. Finally, the EEFMs were registered with the percolated solutions.

One test set of samples was prepared to evaluate second-order advantage achievement, including some potential interferences. Considering that glyphosate is widely used in farm environments, degradation products, and other chemicals commonly used to control pests in plants and livestock could be present. In this way, sixteen solutions containing AMPA and carbofuran were prepared with concentrations of glyphosate in the range of 0–90 µg $L^{-1}$ according to a central composite design.

### *3.4 Real samples*

Different water samples commonly used for agricultural purposes were studied. Then, tap and lagoon water samples were collected from the Valparaiso drinking water system (Curauma, Valparaiso) and Light's lagoon (Curauma, Valparaíso), respectively. Besides, samples of well waters were taken from Casablanca village (Valparaiso, Chile) and Rio Blanco (Los Andes, Valparaíso, Chile), respectively. All samples were filtered using a nylon membrane (0.22 µm) and stored at 4 °C until analysis.

For the analysis, each sample was fortified with a known concentration of Glyphosate, reaching concentrations ranging between 0 and 150 µg L-1. Then, these spiked samples were subjected to the same procedure described in section 2.3.

## 4. Results and discussion

### *4.1. Fluorescence response for glyphosate*

Glyphosate presents a simple chemical structure with no native fluorescence. Then, glyphosate can be derivatized by reacting in alkaline media (pH: 9.0-9.5) with an excess of fluorogenic reagents, such as NBD-Cl and FMOC-Cl [17]. Using NBD-Cl, the acidification of this solution improves the sensitivity, while for FMOC-Cl the fluorescence is evaluated in the same alkaline medium [32, 40]. This study evaluated different pH values during the derivatization process and before registering the fluorescent signal. Nevertheless, no significant improvements were obtained with pH conditions different from the reported procedures. In the same way, no significant improvements were observed in emission fluorescence with the addition of different surfactants (Triton X 100, CTAB, and SDS).

The EEFMs obtained for a typical standard solution of glyphosate using NBD-Cl and FMOC-Cl as fluorogenic reagents are presented in Fig. 1A and Fig. 1B, respectively. As can be seen, the glyphosate derivative formed with FMOC-Cl shows an emission maximum centered at 320 nm, while the derivative produced with NBD-Cl has a maxima emission close to 530 nm. These wavelengths agree with previous reports [32, 41]. In addition, the excitation and emission spectra highly overlap to the glyphosate derivative when NBD-Cl is used (see Fig. 1C). In contrast, the FMOC-Cl derivative shows the same fluorescence maxima as

free fluorogenic agent (see Fig. 1D). The variation in photophysical behavior can be associated to variations in the glyphosate bonds formed with each fluorogenic molecule and their potential influence on the fluorescent response of the fluorophore moiety. [17, 42]. From an analytical point of view, both situations make the quantification of glyphosate by univariate calibration unviable. In addition, the excess of FMOC-Cl must be eliminated before fluorescence measurements to reach one reliable concentration of glyphosate.

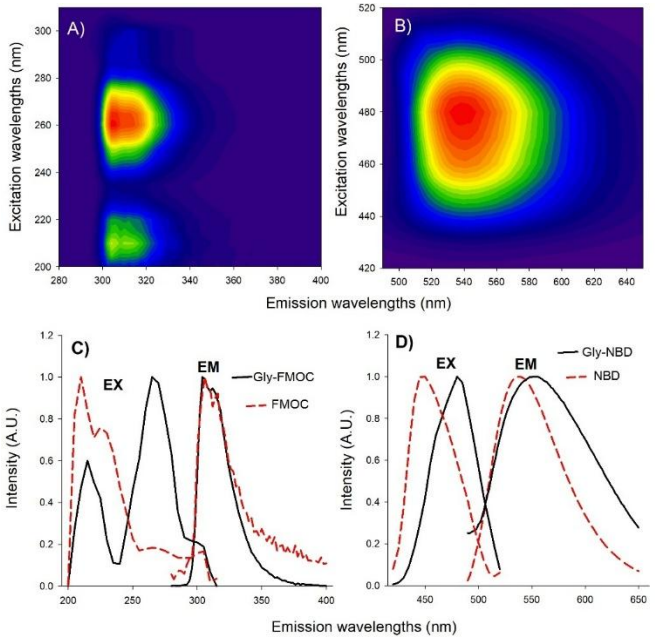


**Figure 1**. Excitation-emission fluorescence matrices for Gly/NBD derivative by using A) FMOC-Cl and B) NBD-Cl reagents. Normalized emission and excitation spectra for fluorogenic agents and Gly-derivatives when C) FMOC-Cl and D) NBD-Cl reagents were used.

*4.2 Calibration and validation set analysis*

As previously shown, the determination of glyphosate in aqueous solutions using univariate calibration is not feasible due to the significant overlap in the fluorescence spectra of fluorogenic agents with their corresponding glyphosate derivatives. To evaluate the analytical capabilities of second-order algorithms in overcoming this challenge, we employed Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) and Unfolded Partial Least Squares coupled with Residual Bilinearization (U-PLS/RBL) to model the excitation-emission fluorescence matrices.

Table 1 presents the figures of merit obtained with both algorithms using FMOC-Cl and NBD-Cl fluorogenic agents. As can be seen, higher sensitivity was obtained using NBD-Cl in the calibration set, probably due to less spectral overlapping in both spectral dimensions. In addition, the lower detection limits were reached when the UPLS-RBL algorithm was used. In this case, this flexible model based on latent variables showed higher efficiency in obtaining quantitative information, probably due to the difficulties of MCR-ALS in obtaining reliable solutions to data decomposition. Other constraints and initialization alternatives were evaluated, without significant improvements. An independent set of Gly solutions with different concentrations to the calibration set, named the validation set, was analyzed to demonstrate the predictive capacity of the model. Similar prediction errors were obtained when NBD-Cl was used for both algorithms. The particular interest resulted the satisfactory figures of merit obtained for FMOC-Cl, including lower prediction error and detection limit, probably due to better modelling of this purified medium resulting from the clean-up process applied to remove the excess of FMOC-Cl reagent.

One test set of Gly solutions containing potential interferences, such as AMPA and carbofuran, was analyzed to determine whether these models achieved the second-order advantage. Both compounds can potentially interfere with the Gly response due to their chemical similarities with this analyte. As shown, both interferences (see Figure 2B and 2C) exhibit a spectral fluorescence response different to NBD-Cl (see Figure 2A) and Gly/NBD derivative (see Figure 1B), showing significant overlap with the spectrum of this analyte. In contrast, when FMOC-Cl is used, both interferences displayed a similar spectral response to Gly, highlighting difficulties in quantifying Gly in the presence of these compounds. As observed in Table 1, the prediction error is considerably higher when this fluorogenic agent is used. In contrast, a lower prediction error is obtained with NBD-Cl as a fluorogenic agent and the UPLS-RBL algorithm. This can be explained by the higher resolution previously evidenced with this algorithm.

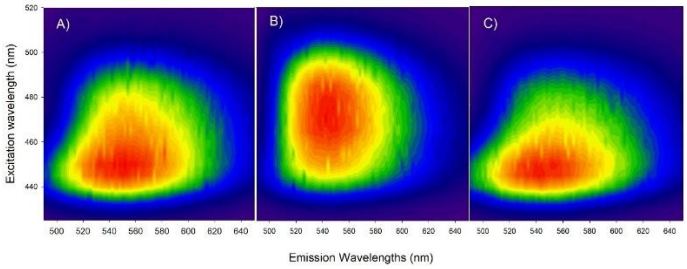


**Figure 2**. Contour plot for emission-excitation fluorescence matrices obtained for A) NBD-Cl reagent, B) AMPA-derivative and C) Carbofuran-derivative using NBD-Cl as fluorogenic agents.

Finally, despite FMOC-Cl combined with UPLS-RBL offering better figures of merit for the calibration set, this combination appears inadequate for predicting Gly concentrations in the presence of unexpected interferences. In contrast, applying NBD-Cl with this algorithm offers a better alternative to Gly quantification in real sample scenarios. For this reason, only this approach will be considered for the analysis of real samples.

**Table 1.** Figures of merit for the determination of glyphosate by using FMOC-Cl and NBD-Cl as fluorogenic agents.

| | | ***U-PLS/RBL*** | | ***MCR-ALS*** | |
|---|---|---|---|---|---|
| | **Units** | **FMOC-Cl** | **NDB-Cl** | **FMOC-Cl** | **NDB-Cl** |
| **A) Calibration** | | | | | |
| Number of factors | - | 3 | 2 | 3 | 2 |
| LOD[A] | $\mu g\ L^{-1}$ | 0.3 – 2.8 | 0.3 – 4.4 | 19 | 8.6 |
| LOQ[B] | $\mu g\ L^{-1}$ | 1.0 - 8.3 | 11.2 – 13.1 | 57 | 26 |
| Sensitivity | $FU^{C}L\ \mu g^{-1}$ | 9.6 | 25.7 | 2.5 | 21 |
| RMSEC[D] | | 1.1 | 2.2 | 6.5 | 3.0 |
| REP (%)[E] | | 1.4 | 2.0 | 8.6 | 4.1 |
| **B) Validation set** | | | | | |
| RMSEP[F] | $\mu g\ L^{-1}$ | 20.1 | 2.1 | 12.8 | 2.3 |
| REP[G] | % | 13 | 1.4 | 9 | 3.6 |
| **C) Test set** | | | | | |
| RMSEP[F] | $\mu g\ L^{-1}$ | 68.5 | 8.3 | 93.4 | 46 |
| REP[G] | % | 45.6 | 17.1 | 62.1 | 32.2 |

[A] LOD: Detection limit; [B] LOQ: Quantification limit; [C] FU: Fluorescence units; [D] RMSEC, Root mean square error of calibration, and [F]RMSEP: root-mean-square error of prediction were calculated in according with $RMSE=[(1/I)\sum_1^I (c_{nominal}-c_{predicted})^2]^{1/2}$ where I is the number of prediction samples and $c_{nominal}$ and $c_{predicted}$ are the actual and predicted concentrations, respectively. [E]REC: Relative error of prediction, $REP=100*RMSEP/\bar{c}$ where $\bar{c}$ is the mean calibration. The validation set corresponds to Gly solutions with different concentrations to calibration set. The test set corresponds to Gly solutions containing unexpected interferences (see text for more details).

**Note**: Sensitivity, LOD, and LOQ were calculated according to reference [38].

*4.3 Analysis of real samples*

The proposed method was applied to different natural waters, including tap, lagoon, river, and well samples. As Gly is undetectable in analyzed samples, these samples were fortified with this analyte at five different concentration levels. In general, a significant matrix effect was identified during the analysis of these samples, requiring calibration on the matrix. This fact was probably due to the influence of sample components on the Gly-derivatization reaction, as observed in previous reports[33].

The typical EEFMs obtained for the water samples analyzed in the absence of Gly are presented in Figure 4. As can be seen, the presence of spectral interferences is evident, making it mandatory to use second-order multivariate methods to quantify Gly in these samples.

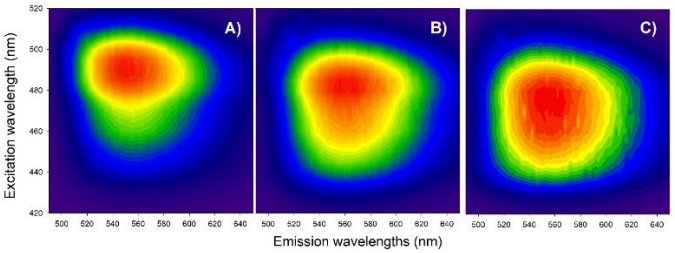


**Figure 3**. Typical excitation-emission fluorescence matrices for NBD-Cl reagent without Gly addition for A) Lagoon, B) Well and C) river samples.

In general, these samples required between 3 and 5 components to achieve a good correlation ($R^2 > 0.99$) between real and predicted concentrations, evidencing the complexity of the samples in relation to the validation set. As shown in Table 2, the detection limits and prediction errors obtained for the real samples are similar to those obtained for the calibration samples, demonstrating the robustness of the proposed method. In addition, as shown in Figure 4A, the predicted values agree with the nominal values despite the higher complexity of the real samples, demonstrating the analytical potential of the proposed method.

The elliptical joint confidence region (EJCR) test was conducted to evaluate the accuracy of the predicted concentrations [43]. In our case, the obtained ellipses (Figure 4B) include the theoretically expected values of slope = 1 and intercept = 0, indicating the accuracy of predicted concentrations.

In summary, these findings showcase the analytical effectiveness of the proposed method for monitoring glyphosate levels in natural waters.

**Table 2**. Figures of merit for the determination of glyphosate in real water samples.

| | | Samples | | | |
|---|---|---|---|---|---|
| **Parameters** | **Units** | **Tap water (M1)** | **Lagoon water (M2)** | **Well water (M3)** | **River water (M4)** |
| **Components** | | | | | |
| **LOD**[A] | µg $L^{-1}$ | 0.02 - 1.3 | 0.02 - 1.3 | 0.03 – 1.0 | 0.02-2.6 |
| **LOQ**[B] | µg $L^{-1}$ | 0.05 - 3.9 | 0.05 – 3.9 | 0.09 – 2.9 | 0.05-7.7 |
| **RMSEP**[C] | µg $L^{-1}$ | 3.1 | 0.53 | 0.35 | 0.94 |
| **REP**[D] | % | 4.1 | 0.68 | 0.45 | 1.2 |

[A] LOD: Detection limit; [B] LOQ: Quantification limit; [C] RMSEP: root-mean-square error of prediction; [D] REP: relative error of prediction.

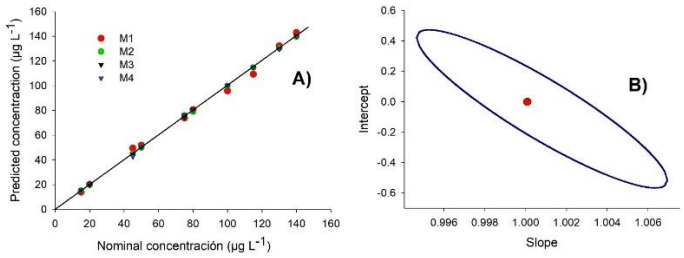


**Figure 4**. (A) Plot of the UPLS-RBL-predicted concentrations of deltamethrin as a function of nominal values in fortified natural water samples. (B) Regions of the elliptical joint (at a 95% confidence level) for the slope and intercept of the regression regarding fortified natural water samples. The red circle marks the theoretical point (intercept: 0, slope: 1). **Sample correspondence**. M1: well water; M2: lagoon water; M3: river water; and M4: tap water.

## CONCLUSION

The derivatization of Gly with fluorogenic agents, coupled with fluorescence spectroscopy and multivariate calibration, enabled the proposal of an analytical method for quantifying this compound. Different fluorescent labelling and second-order algorithms were evaluated, with NBD-Cl coupled to the UPLS-RBL algorithm evidencing the lowest prediction errors, even in the presence of unexpected interferences, showcasing the achievement of the second advantage. Overall, the proposed method enabled the detection of Gly at a concentration level of µg $L^{-1}$ with a prediction error of less than 5%. Finally, the method was applied to the analysis of fortified real water samples, yielding prediction errors of less than 5%. All these results demonstrate the analytical potential of the proposed method for monitoring Gly in the hydrological cycle.

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