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Fast Kinetic Determination of 1-Naphthylacetic Acid in Commercial Formulations, Soils, and Fruit Samples Using Stopped-Flow Phosphorimetry

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A kinetic method has been developed for the determination of 1-naphthylacetic acid by means of micellar-stabilized room temperature phosphorescence (MSRTP) using the stopped-flow mixing technique. The main feature of this system is that it diminishes the time required for the deoxygenation of the micellar medium and for the phosphorescence development. Phosphorescence enhancers such thallium(I) nitrate, sodium dodecyl sulfate (SDS), and sodium sulfite were optimized to obtain maximum sensitivity. The pH was also optimized as it strongly affects the luminescent properties of 1-naphthylacetic acid. A pH of 6.6 was selected as adequate for the phosphorescence development. The kinetic curve of 1-naphthylacetic acid phosphorescence was scanned at $\lambda_{ex} = 278$ nm and $\lambda_{em} = 490$ nm, and the maximum rate of phosphorescence was taken as the analytical signal. This was obtained by calculating the maximum slope of the curve in an interval of 3.6 s as it provided a good noise-to-signal ratio. This method permitted the determination of 1-naphthylacetic acid throughout a concentration range of 100−1800 ng mL^{-1} with high precision (relative standard error = 0.91% and relative standard deviation = 2.30%; 1-naphthylacetic acid concentration = 800 ng mL^{-1}). According to the Clayton criterion, the detection limit was 45 ng mL^{-1}. The same limit resulted in 39.3 ng mL^{-1} when the error propagation theory was applied. The applicability of the method was successfully demonstrated by determining 1-naphthylacetic acid in different kind of samples, such as phytosanitary products, soils, pears, and apples. Recovery values not significantly different from the nominal content or the spiked amount were found for these determinations.

KEYWORDS: Naphthylacetic acid; phosphorescence; stopped flow; soil; fruit

INTRODUCTION

1-Naphthylacetic acid (**Figure 1**) is a naphthalene derivative widely used in the world of farming. It is applied as a biocide for the control of fungus on fruit. In addition, it is a growth regulator, and so it has a general application to prevent the preharvest dropping of apples, mangoes, etc. It is also used in combination with other chemicals as a thinning fruit agent by spraying. For technical reasons, such as the avoidance of persistence or its toxicity to mammals or birds, it is advised that this product be applied to crops at low concentrations (*1*−*3*). Thus, only trace amounts in soil and fruit might be expected arising from the agricultural use of 1-naphthylacetic acid products, which makes its control far more problematic, and so the development of selective and sensitive analytical methods is required.

It is possible to find several papers in the bibliography proposing different analytical methods for the determination of 1-naphthylacetic acid in commercial formulations, water, soil, and vegetables. These methods include spectrofluorometry (*4*−*8*), high-performance liquid chromatography (*9*−*13*), gas chromatography (*14*) and its combination with mass spectrometry (*15*), and room temperature phosphorescence (RTP) induced by β-cyclodextrin or using a micellar agent (*16*−*21*). Although these techniques cover a wide range of analytical procedures, none of them is based in its kinetic determination, which makes its rapid analysis and automation possible.

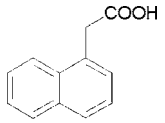

Figure 1. Chemical structure of 1-naphthylacetic acid.

Although the use of solid supports in RTP has the limitation of the presence of background signals (which limits its application to trace analysis) and the critical sample preparation and measurement requirements (which strongly affects the result obtained), application of the stopped-flow mixing technique for fast acquisition of analytical data in micelle-stabilized (MS) RTP

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is as a powerful tool for avoiding those drawbacks (*22*). This methodology, applied to the determination of 1-naphthylacetic acid in this paper, provides great results when fast reactions are involved in the measurement process as it mixes together the sample and reagent solutions. In addition to a reliable means of automating kinetic methods for routine analyses by the controlled mixing of the sample and reactants, this technique overcomes some problems arising from the instability of the reaction products and, also, the interactions between the excess of reagents and the reaction products, in contrast to conventional or even-flow techniques.

The combination of stopped-flow mixing technique and room temperature phosphorescence is of recent application (*22*−*27*). This can be used to develop both a kinetic determination method by measuring the slope of the kinetic curve and equilibrium methods by measuring the amplitude of the kinetic curve, which is directly proportional to the analyte concentration. Panadero et al. (*22*) reported on the determination of carbaryl for the first time. Later on, the same authors applied the technique to the analytical determination of naproxen (*23*), and it has since been further developed by several authors (*24*−*27*).

This paper reports on the application of the combination of stopped-flow mixing technique and room temperature phosphorescence to the determination of 1-naphthylacetic acid in commercial formulations and soil, apple, and pear samples.

The resulting method is sensitive and precise and allows the determination of 1-naphthylacetic acid in only 75 s.

EXPERIMENTAL PROCEDURES

Caution. Thallium salts are very toxic when inhaled, ingested, or absorbed through the skin.

Apparatus. The phosphorimetric measurements were performed on an Aminco Bowman series 2 luminescence spectrometer, connected to a microcomputer with AB2 software, which runs on the OS2 operating system. The instrument utilizes a 7 W integral pulsed xenon lamp for phosphorescence measurements. To measure kinetic luminescence reactions, the instrument incorporates a MilliFlow stopped-flow reactor, which consists of two fill syringes, two drive syringes, a mixing and observation cell (2 mm of path length), a stop syringe, a stop block, an exhaust, and fill valve levers. This reactor allows the study of changes in luminescence reactions when two reactants are vigorously mixed and suddenly stopped in the observation cell.

Hamilton gasting syringes of 2.5 mL (drive syringes) were used to contain the two reactant solutions. The syringes are made of controlled, inner diameter, borosilicate glass with precision machined Teflon plunger tips (these pistons are simultaneously driven by air-operated plunger) (**Figure 2**). Thermostatic equipment permits a constant temperature of 20 °C to be maintained in the MilliFlow stopped-flow reactor.

A Crison model 2001 pH meter with a glass-saturated calomel combination electrode was used to measure the pH of solutions.

Software. The AB2 program allows file management, defines parameters for the acquisition mode, and sets up the acquisition parameters to obtain excitation and emission spectra and kinetic curves. The kinetic curve processing was performed by means of the SLOPES program. This program was developed by us and gives the maximum reaction rate by applying least-squares regression to the kinetic curve. We also developed the LSWR program to perform the statistical analysis, and it includes the following options: least-squares regression with replicates; limits of detection assuming the error propagation theory (*28*, *29*) and by the Clayton criterion (*30*); linearity test of ANOVA; precision of the method by means of the confidence bands of the calibration line; homoscedasticity and heteroscedasticity criteria; and the true region for the slope and intercept.

Reagents. All experiments were performed with analytical reagent grade chemicals, pure solvents, and Milli-Q purified water. 1-Naphthylacetic acid was obtained from Aldrich. Sodium dodecyl sulfate (SDS) was obtained from Sigma, and thallium(I) nitrate and sodium sulfite were obtained from Merck. Sulfuric acid was supplied by Panreac.

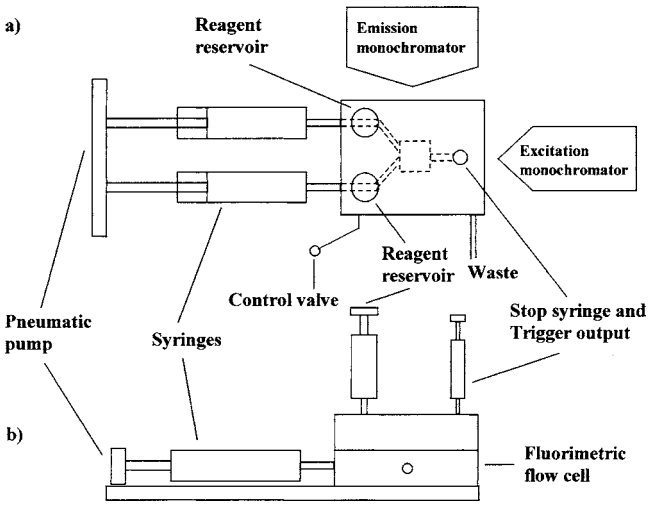

Figure 2. (a) Top and (b) side views of the apparatus.

A stock solution of 1-naphthylacetic acid (10.0 mg dissolved in 100 mL of 0.2 M SDS) was diluted to prepare working standard solutions. This stock standard solution of 1-naphthylacetic acid was stored at room temperature and protected from light. In these conditions, it was stable for at least 4 weeks. The working standard solution of 1-naphthylacetic acid was stable for at least 2 days at room temperature.

Stock standard solutions of 0.2 M SDS, 0.25 M thallium(I) nitrate, and 0.02 M sulfuric acid were used. A 0.25 M sodium sulfite solution was prepared daily.

The commercial technical phytosanitary formulation of Etifix, made by ETISA (Especialidades técnico industriales, S.A., Barcelona, Spain) and supplied by Nufarm (Nufarm España S.A., Barcelona, Spain), was labeled as "1% of 1-naphthylacetic acid".

General Procedure. One syringe (A) of the two drive syringes of the stopped-flow reactor is filled with an aqueous solution containing 25 mM thallium(I) nitrate, 64 mM SDS, 16 mM sodium sulfite, and 4 mM sulfuric acid. The other syringe (B) is filled with a determined volume of the standard solution of 1-naphthylacetic acid so as to result in a final concentration between 100 and 1800 ng mL^{-1} and as much SDS as required to give a 64 mM concentration. In each run, the same volume of each solution is mixed; thus, the final concentration of reactants was just half of the initial except for SDS, which was injected in the same concentration from the two syringes.

The instrument is set up to acquire kinetic curves as follows: λ_{ex} = 278 nm, λ_{em} = 490 nm, band-pass = 16 nm, detector voltage = 1100 V, flash lamp in phosphorescence mode <200 μs (PMT masked) with a 100 μs delay after flash, 800 μs gate width, 5 ms time between flashes, 0.2 s of resolution, and 120 s of measurement time. Then, three replicates of the time trace scan are recorded, and the average is calculated and smoothed. The result is the kinetic curve. To obtain the maximum rate, the file is exported in ASCII mode and the SLOPES program is run after 3.6 s has been selected as interval (which corresponds to 19 experimental data). The content of 1-naphthylacetic acid is determined by using the appropriate calibration graph.

Procedure for Commercial Technical Formulations. For determination of 1-naphthylacetic acid in Etifix (the commercial phytosanitary product studied), 0.1 g of the sample is dissolved in 100 mL of 0.2 M SDS. Suitable volumes of this micellar solution are placed in 25 mL volumetric flasks to obtain in the mixing chamber final concentrations within the calibration graph. These sample solutions, containing the SDS concentration previously fixed (64 mM), are inserted in syringe B, and the general procedures for syringe A and for the determination are followed.

Procedure for Soil Samples. The proposed method was applied to soil samples taken from different sites within Ciudad Real province (Spain). The samples were scooped out of the farming soil at 5-cm depth, put in polyethylene bags, and transported to the laboratory. To determine 1-naphthylacetic acid, the samples are air-dried and the

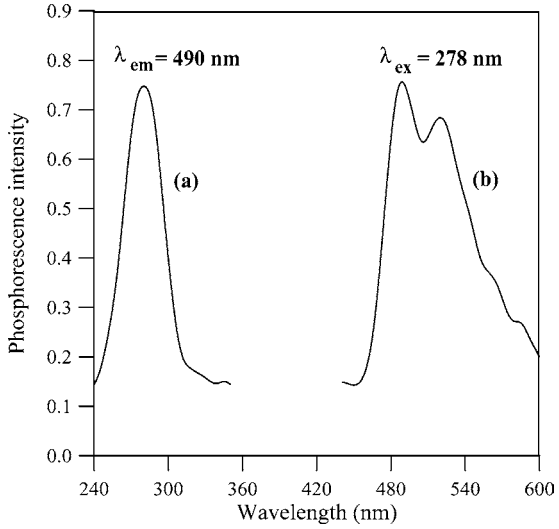

Figure 3. Room temperature phosphorescence spectra of 1-naphthylacetic acid (1000 ng mL^{-1}): (a) excitation spectrum; (b) emission spectrum. Photomultiplier voltage = 1100 V.

fraction of particle diameter <2 mm is separated by sifting. The samples are crushed with an agate mortar.

The soil samples (10 g) are spiked with an exactly known quantity of 1-naphthylacetic acid (ethanolic solution) in such a way that the final concentration is included in the calibration graph. The samples are left at room temperature for 24 h to ensure the binding of the compound to the matrix. The sample is reextracted twice with 30 mL each of chloroform in acidic medium (1 mL of 10% sulfuric acid is added for each extraction process) and filtered on a vacuum. The dry residue is washed with 10 mL of chloroform. All of these extracts are mixed, and the organic phase is evaporated to dryness. The residue is dissolved and diluted to 100 mL with 0.2 M SDS, and then the general procedure is followed.

Procedure for Apple and Pear Samples. A weighed amount of a fruit sample (apple or pear) is chopped, blended, and triturated to form a homogeneous mixture. Fifty grams of the resulting aqueous slurry is transferred in a 250 mL beaker, and a known quantity of 1-naphthylacetic acid (ethanolic solution) is added in such a way that the final concentration is included in the calibration graph. To each slurry are added, with careful shaking, 80 mL of chloroform and 1 mL of 10% sulfuric acid, and the mixtures are shaken for 15 min before vacuum filtering; the residues are washed with 10 mL of chloroform. The combined extract is shaken with 50 mL of 4% sodium hydrogen carbonate solution in a separating funnel, and the organic extract is discarded after separation. The aqueous layer is acidified with 10% sulfuric acid to pH ~2 and then extracted twice with 25 mL portions of chloroform. The organic extract is evaporated to dryness. The residue is dissolved and diluted to 100 mL with 0.2 M SDS, and then the general procedure is followed.

RESULTS AND DISCUSSION

Spectral Characteristics. Figure 3 shows the phosphorescence excitation spectrum at the emission wavelength of maximum intensity, that is, λ_{em} = 490 nm, and the phosphorescence emission spectrum at 278 nm of excitation wavelength of 1-naphthylacetic acid in micellar medium in the previously established optimal conditions. To study the room temperature phosphorescence kinetic curve, the maximum at λ_{ex} = 278 nm and λ_{em} = 490 nm was chosen.

Factors Affecting Phosphorescence. The development of a phosphorescence signal of a dissolved substance requires the following: protective screening effect of micelles for avoiding collisional quenching; the removal of dissolved oxygen from the solution, which is an extremely highly effective phosphorescence quencher and thus has severely restricted the utilization of phosphorescence in analytical chemistry; and the presence of heavy atoms to increase the phosphorescence yield by effectively promoting the $S_1 \rightarrow T_1$ intersystem crossing via spin−orbit coupling (*22*). In this work, we used sodium sulfite to remove oxygen from solutions, SDS as micellar agent, and thallium(I) nitrate as heavy atom. A sharp increase in phosphorescence was obtained in a short time by applying the stopped-flow mixing technique. This is due to the fact that the fast mixing of the stream flow in the cell favors the interaction of oxygen molecules with sulfite ions and their removal.

The general shape of the profile's phosphorescence intensity versus time exhibits three clearly distinguished regions (*31*): The first shows no phosphorescence and corresponds to the time required for consumption of only the oxygen dissolved in the bulk phase where the anion sulfite resides. Dissolved oxygen existing in the micelles quenches the phosphorescence. The second region starts with the diffusion of the oxygen out of the micelle pseudophase, reaching the bulk phase where it reacts with sulfite. The rate of diffusion of the oxygen limits the efficiency of the quenching and, therefore, a gradual increase is observed in the phosphorescence development. Finally, a steady-state region is reached, in which all of the oxygen dissolved in the micelle has diffused out and been reduced with sufite in the bulk phase. Consequently, phosphorescence is practically constant with time variations in this latter region.

Variables affecting the performance of the proposed kinetic method for the determination of 1-naphthylacetic acid were studied and optimized in order to obtain the maximum rate of the luminescence reaction. All stated concentrations were initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing) except for SDS, which was injected in the same concentration from the two syringes. Each kinetic result was the average of three scans.

The effect of the sodium sulfite concentration on the system was studied by preparing samples in which the sodium sulfite concentration was varied from 8 to 28 mM. It was observed that an excess of sodium concentration produced intensity decay. This was also observed for the rate of phosphorescence. This fact was interpreted by Díaz García et al. (*31*) as the displacement of thallium(I) by sodium from the micelle surface. After the phosphorescence kinetic curves of these solutions had been studied, it was concluded that a concentration of 16 mM sodium sulfite was appropriated to eliminate oxygen completely from the solution while producing an adequate curve of phosphorescence.

The influence of pH on the phosphorescence development was studied by adding different amounts of H_2SO_4 to syringe A. **Figure 4** shows the slope of kinetic curves at different pH values and the time required for those pH values to obtain the maximum and stable room temperature phosphorescence signal. As it can be readily noted, the phosphorescence development was strongly affected by the acidity of the medium. No phosphorescence was observed pH values <6.0. The highest slope and the shortest time were observed at a pH of 6.6. Hence, this pH value was selected as adequate for the determination. This pH corresponds to the addition of 4 mM H_2SO_4 to the solution.

It was also determined how the slope of kinetic curve varied with changes in thallium(I) nitrate concentration. The tested concentration ranged from 10 and 35 mM. **Figure 5** shows how the slope increases with increase in thallium(I) concentration up to 25 mM. Then a drastic decrease is observed. On the other hand, the time required for phosphorescence development diminishes up to 25 mM, and a great increase is observed at

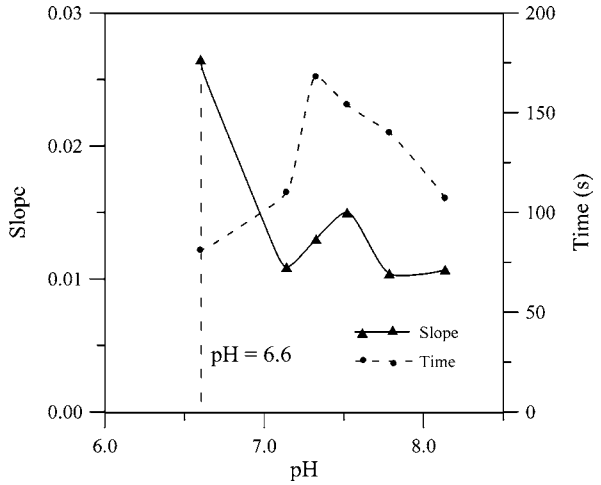

Figure 4. Effect of pH on (▲) the rate of phosphorescence and (●) the time required to obtain a stable room temperature phosphorescence signal.

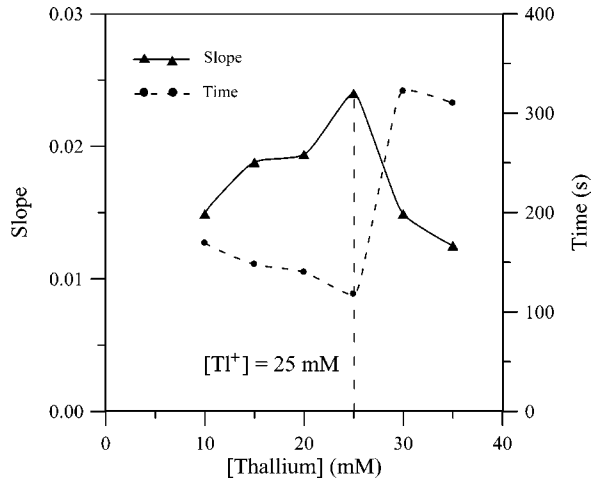

Figure 5. Effect of thallium(I) concentration on (▲) the rate of phosphorescence and (●) the time required to obtain a stable room temperature phosphorescence signal.

higher concentrations. Thus, a 25 mM thallium(I) concentration was selected for the determination.

The effect of surfactant concentration was investigated by preparing samples of SDS concentrations ranging from 52 to 72 mM. A 64 mM concentration of SDS was chosen because a slight diminution in the time required for phosphorescence development was observed, although no significant changes were observed in the slope of the curve.

The influence of 1-naphthylacetic acid concentration on the phosphorescence was studied under those conditions. The rate of phosphorescence was found to be linearly dependent on 1-naphthylacetic acid concentration up to 1800 ng mL^{-1}. Hence, the 1-naphthylacetic acid calibration was carried out for concentrations up to 1800 ng mL^{-1} with three replicates per point.

Determination of 1-Naphthylacetic Acid and Validation of the Method. Under the optimum chemical and instrumental conditions previously specified, we propose a method to determine 1-naphthylacetic by studying the phosphorescence curve development. This was scanned at an emission wavelength of 490 nm and an excitation wavelength of 278 nm. The calibration graph was constructed with three replicates per point, in the concentration range of 100−1800 ng mL^{-1}, by measuring the maximum reaction rate of the phosphorescence development. This was worked out after fitting of the experimental data by least-squares regression for an interval of 3.6 s. This mathematical operation was performed by means of the SLOPES program. It was assumed that the obtained slope corresponded to the maximum rate of phosphorescence development.

Table 1. Results from Statistical Analysis of 1-Naphthylacetic Acid Calibration in 0.2 M SDS: Least-Squares Regression with Three Replicates per Point[a]

intercept on ordinate (a)	9.79×10^{-3}
SD of intercept on ordinate (S_a)	2.9×10^{-4}
slope (b)	3.38×10^{-2}
SD of slope (S_b)	2.7×10^{-4}
SD of regression (S_r)	8.2×10^{-4}
determination coefficient (r^2)	0.998
confidence interval of intercept	1.04×10^{-2}, 9.20×10^{-3}
confidence interval of slope	3.43×10^{-2}, 3.32×10^{-2}

[a] Theoretical t value ($\alpha = 0.05$, $n = 28$) = 2.05.

The proposed method, maximum rate measurements versus concentration, was evaluated by a statistical analysis of the experimental data by fitting the overall least-squares line according to $y = a + bx$ (*32*). **Table 1** shows the outstanding results from the statistical analysis of 1-naphthylacetic acid with three replicates per point. It being accorded to the random residual distribution obtained, it was concluded that the calibration line abided homoscedasticity criteria; that is, the variance was constant over the entire dynamic range and independent of the concentration. Thus, it was accepted that the rate of phosphorescence was linearly proportional to 1-naphthylacetic acid concentration, in the concentration range studied.

The IUPAC (*33*) proposes a detection limit in which only the standard deviation of the blank is considered. However, if the theory of error propagation is considered, the value of the detection limit accounts for the reliability of the blank measurements and the signal measurements of the standards (*28*). Eventually, Clayton (*30*) considers the probability of false positives and false negatives. On the basis of these premises, we obtained detection limits of 30.5, 39.3, and 45 ng mL^{-1} ($\alpha = \beta = 0.05$; $n = 30$) for the IUPAC recommendations, the error propagation theory, and Calyton's criterion, respectively.

To determine the repeatability of the recommended procedure, a series of 10 standard samples containing 800 ng mL^{-1} of 1-naphthylacetic acid was prepared, and the analytical signal was tested in all of these samples. When the theory of error propagation (95% confidence level) was applied to the calibration graph, a relative standard error of 0.91% was found. The relative standard deviation of replicates was 2.30%.

Finally, to validate the method, comparative determinations of 1-naphthylacetic acid were carried out. In this assay, 10 different concentration standards of 1-naphthylacetic acid were analyzed by applying the method we are proposing and also by an already accepted method (*12*). The best procedure to establish the comparison consists of calculating the sample concentrations in reverse through both methods, which should lead to exactly the same concentration values for each sample. Therefore, if a least-squares regression of the concentration through the proposed method versus concentration through bibliographic method is done, a null intercept on the y-axis and a unitary slope will be worked out (*34*). To adequately interpret the regression results, it is necessary to consider how the presence of errors affects the test. Whereas the proportional systematic error leads to a change in b and the constant systematic error gives an intercept different from zero, random errors lead to a distribution of the points around the least-squares line, and, as a result, the slope and intercept are not significantly different

Table 2. Recovery of 1-Naphthylacetic Acid in Real Samples

sample	concn added or certified	concn found ± s[a]	mean recovery (%)
commercial fungicide			
Etifix	10.0 mg g^{-1}	10.40 ± 0.02 mg g^{-1}	104.0
soils			
sample 1	30.0 μg g^{-1}	28.30 ± 1.58 μg g^{-1}	94.3
sample 2	30.0 μg g^{-1}	28.92 ± 2.25 μg g^{-1}	96.3
apples	10.0 μg g^{-1}	9.71 ± 0.44 μg g^{-1}	97.1
pears	10.0 μg g^{-1}	9.63 ± 0.37 μg g^{-1}	96.3

[a] Mean ± *s* for three replicates.

from unity and zero, respectively. The result was successful for the validation of the method at a 95% confidence level, and the resulting determination coefficient was 0.994.

The above procedure was also applied to the determination of six different concentration levels of 1-naphthylacetic acid in a phytosanitary product (Etifix), pear, and one of the soils under study. The results were likewise successful with intercepts on the *y*-axis and slopes not significantly different from zero and one, respectively, and determination coefficients slightly lower: 0.990, 0.979, and 0.972, respectively.

Determination of 1-Naphthylacetic Acid in a Technical Formulation and Soil, Apple, and Pear Samples. The proposed kinetic method was applied to the determination of 1-naphthylacetic acid in a commercial phytosanitary product, Etifix. The samples were prepared as described under Experimental Procedures. The recovery achieved by means of the proposed method agrees well enough with the nominal content of 1-naphthylacetic acid in the commercial preparation (**Table 2**).

The proposed method was also applied to soil samples taken from different sites within Ciudad Real province (Spain) and also Spanish agricultural products such as apples and pears. All of these samples either were free of 1-naphthylacetic acid contamination or perhaps were contaminated at concentrations lower than the detection limit. Therefore, a recovery study was carried out with standard solutions of 1-naphthylacetic acid.

The soil samples (10 g) were spiked with 1-naphthylacetic acid and then shaken for 45 min with 60 mL of 0.2 M SDS. The suspensions were centrifuged and filtered to remove the soil particles. A portion of the separated micellar phases was analyzed following the kinetic method described above. A strong quenching effect was observed, which was probably caused for the presence of ionic compounds in the soil samples. Therefore, a strategy based on the prior extraction of 1-naphthylacetic acid was developed to avoid matrix interference. The high solubility of 1-naphthylacetic acid in chloroform permitted extraction of this compound as described under Procedure for Soil Samples. The recovery ratios, of known amounts of 1-naphthylacetic acid added to two different soil samples, were obtained by using calibration graphs (rate measurements versus concentration). The results obtained showed good agreement with the amount added to each sample (**Table 2**).

The apple and pear samples were spiked with the fungicide, and the determination of 1-naphthylacetic acetic was carried out by applying prior extraction with chloroform. Nevertheless, the phases showed slight turbidity, which was attributed to the matrix effect. For this reason, a re-extraction process to aqueous phase using sodium hydrogen carbonate solution was applied. Eventually, a new extraction with chloroform, as described under Procedure for Apple and Pear Samples, was realized. The recovery percentages for apple and pear samples are summarized in **Table 2** and are much better than those described by Maiti et al. (*12*).

Conclusions. The proposed method shows several advantages. First, as has been demonstrated throughout this paper, the combination of MSRTP with the stopped-flow mixing technique is a rapid method for the determination of 1-naphthylacetic acid, because it is not necessary to measure the analytical signal in equilibrium conditions. Besides, when it is compared to conventional MSRTP, phosphorescence development takes less time, and so kinetic data are obtained much more quickly. Second, as it is a flow system, it could easily be automated, thus making a future automatic quality control of this compound easier. Other methods, such as that described by Vilchez et al. (*8*), use solid-phase spectrofluorometry which complicates the method itself and the instrumentation. Third, the proposed method shows a better detection limit (30.5 ng mL^{-1}) than others described in the bibliography (*16*).

Finally, this method shows better recoveries of this growth regulator than others reported in the bibliography. Thus, Maiti et al. (*12*) show a recovery of 85−90% for the 1-naphthylacetic acid added to apples using an HPLC method, and Sigrist et al. (*4*) obtain recoveries of 90−103% in the same type of sample, using a fluorometric method. Using HPLC with fluorescence detection, Archer et al. (*11*) obtained recoveries ranging from 76.1 to 95.5% and a method sensitivity of 0.05 μg mL^{-1}, which have clearly been improved by us. In addition to a better recovery and sensitivity, our method is simpler and less time-consuming than this latter method, which requires that the sample be blended with EtOAc, refluxed for 1 h, then evaporated to dryness, and hydrolyzed for 90 min in 6 N HCl.

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