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Direct Determination of Gibberellic Acid in Tomato and Fruit by Using Photochemically Induced Fluorescence

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ABSTRACT: A simple, sensitive method for determining gibberellic acid based on photochemically induced fluorescence detection was developed to determine this plant growth regulator in a technical formulation, tomato, and fruit samples. The principle for the determination is the photochemical reactivity of the gibberellic acid, being consistent with the occurrence of photoaromatization and photochemical dimerization with loss of carbon dioxide, and with the likely formation of various fluorescent photoproducts. Six min of UV (mainly at 253.7 nm) irradiation in a solution containing 50% (v/v) methanol and buffer at pH 5 provided the best results. The calibration curve was linear over the concentration range 50−150 ng mL^{-1}, and the limit of detection was 1.7 ng mL^{-1}. The method is useful to determine gibberellic acid in samples with background fluorescence such as plum and tomato without the need for labor-intensive preparation as a result of UV irradiation suppressing the fluorescent background.

KEYWORDS: *gibberellic acid, photochemically induced fluorescence, agricultural products*

INTRODUCTION

Plant growth regulators are a class of synthetic pesticides similar in physiological activity to their natural pesticides (plant hormones) that can effectively promote, inhibit, or modify plant growth and development.[1] Plant growth regulators and fertilizers are being increasingly used worldwide, but especially in developing countries, where agriculture plays a crucial role, to maximize crop yields. As a result, their toxicity and increasing presence in residual amounts in foods (particularly fruits) and the environment have raised growing concern in recent years. Plant growth regulators include auxins, gibberellins, cytokinins, and inhibitors. Gibberellic acid, the chief representative of gibberellins, is a compound of especial economic and industrial significance.[2,3]

Gibberellic acids are typically applied to growing crops (field crops, small fruits, vines, and tree fruits), ornamental and shade trees, and ornamental plants, shrubs, and vines. Gibberellic acid has an acute dermal toxicity LD_{50} > 2 g/kg and an acute inhalation-negative LC_{50} at 2.98 mg/L and LC_{50} > 5.9 mg/L; gibberellic acids are in toxicity category III. Humans may be exposed to residues of gibberellic acid through the diet. This has led to the establishment of a tolerated or maximum residue limit (MRL) of 0.15 mg L^{-1} for this compound in citrus fruits, grapes, leafy vegetables, stone fruits, and blueberries.[4]

Quantitative analyses for plant growth regulators are often required in agriculture and plant physiology. Determining phytohormones in plant tissue is usually rather difficult owing to their presence in trace amounts and the coexistence of a wide variety of interferents. This has promoted the use of various extraction procedures including liquid-phase and solid-phase extraction[5] to purify phytohormones from plants prior to their determination. Also, gibberellic acid has been determined by using a variety of techniques including liquid chromatography[5−8] in addition to spectrophotometry, fluorimetry, and sequential injection analysis with potentiometric detection.[9−12]

Photochemical derivatization is a simple, clean, and efficient analytical technique giving strongly fluorescent photoproducts from nonfluorescent or weakly fluorescent analytes.[13] Photochemical reactions, particularly those of the photolytic type, can yield a variety of products. Thus, irradiation of some analytes dramatically reduces or even completely suppresses their native fluorescence. However, the photochemical reactions of many nonfluorescent compounds increase their absorption coefficients and fluorescence quantum yields by an effect of their phototransformation into stable, fluorescent photoproducts. Photochemically induced fluorescence (PIF) is a precise, selective, sensitive analytical technique that is often used in combination with flow injection analysis to determine photoreactive drugs in biological fluids.[14−18] In recent years, PIF has proved an excellent choice for the analysis of various pesticide families (particularly nonfluorescent pesticides) by virtue of its high expeditiousness, low reagent consumption, and the need for no sample dilution.[19−23]

This article reports the determination of gibberellic acid by its photochemically induced fluorescence in a commercial formulation and various agricultural products including tomato and fruits (mandarin orange, plum, and strawberry tree berry). Two of the fruit samples (mandarin orange and strawberry tree berry) possess no native fluorescence or photochemically induced fluorescence; by contrast, plum and tomato exhibit strong fluorescence that disappears upon irradiation. All experimental variables facilitating the observation of fluorescence from gibberellic acid were examined in order to optimize its determination in spiked tomato and fruit samples.

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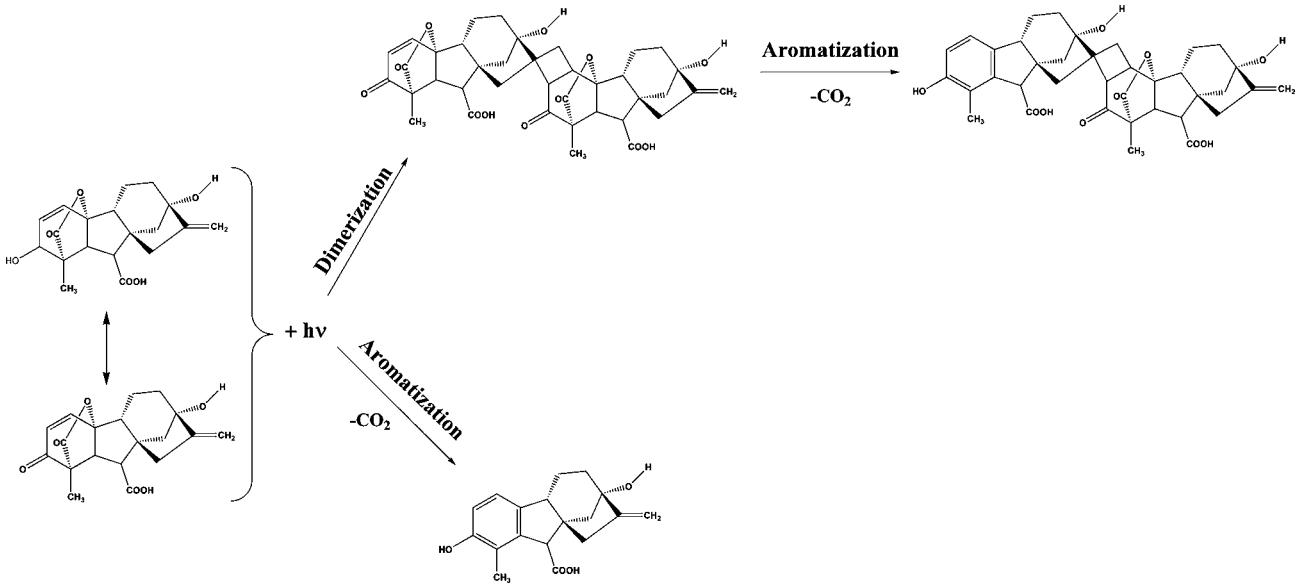

Figure 1. Diagram for the photochemical reactions of gibberellic acid.

EXPERIMENTAL SECTION

Reagents. All of the experiments were performed with analytical reagent grade chemicals, pure solvents, and Milli-Q purified water. The standard gibberellic acid was obtained from Fluka (Seelze, Germany). Hydrochloric acid and sodium hydroxide were obtained from Panreac (Barcelona, Spain). Ethanol, methanol, and sodium acetate were purchased from Sigma-Aldrich (St. Louis, USA).

A stock standard solution of gibberellic acid (200.0 mg L^{-1}) was prepared in ethanol and stored at 4 °C in the dark. Under these conditions, the solution was found to remain stable for at least one month. Working-strength solutions of gibberellic acid were daily prepared by using appropriate aliquots of the stock standard solution, each one remaining stable for at least 4 h at room temperature. A 0.5 mol L^{-1} acetic acid-sodium acetate buffer solution of pH 5 was used. The commercial, technical, and phytosanitary formulation of Clemencuaje, made and supplied by Aventis (Aventis CropScience España S.A., Cheste, Valencia, Spain), was labeled to contain 1.6% gibberellic acid.

Strawberry tree berry, plum, and tomato samples were collected from various plantations in the province of Ciudad Real (central Spain), and mandarin oranges were purchased at a market in the city of Ciudad Real.

Instrumentation and Analytical Procedure. All fluorimetric measurements were made on a Photon Technology International (PTI) Quanta-Master spectrometer equipped with a xenon lamp, Czerny−Turner monochromators on the excitation and emission channels, and a digitally synchronized photomultiplier tube for signal detection. The system was connected to a PC via an ethernet interface and governed by the software Felix32, which allows the analyst to obtain excitation and emission spectra and total fluorescence spectra for processing with various mathematical tools including smoothing, differentiation, derivation, and integration.

The experimental setup comprised a Peltier cuvette holder for quartz cuvettes with a path length of 1.0 cm providing temperature control of the cuvette from −20 to 100 °C. The irradiation system consisted of a PSA 10.570 UV Cracker photoreactor from PS Analytical (Orpington, Kent, UK) equipped with a 4.5 W low-pressure mercury lamp (with main emission at 253.7 nm) and a photoreaction coil in the form of a 4.78 m × 1.56 mm i.d. Tygon PTFE tube helically coiled around the lamp.[24]

Calibration curves were obtained by transferring appropriate aliquots of a standard solution of gibberellic acid to 10.0 mL volumetric flasks and diluting the analyte to a concentration in the range 50−150 ng mL^{-1}. A volume of 0.8 mL of buffer solution (pH 5), 5 mL of methanol (50% v/v), and enough ethanol to have a 5% v/v final content in this alcohol were then added to each flask before filling to the mark with water.

The previous solutions were pumped through the PTFE photoreactor by means of a Gilson Minipuls 3 peristaltic pump operating at 5 rpm under UV radiation for 6 min. Then, a portion of each solution was transferred into a standard 1.0 cm quartz cuvette and placed in the instrument holder at 20 °C. The sensitivity was diminished when the online technique was used because the effective cell path with the flow configuration was too small.

Fluorescence intensities were measured at an excitation wavelength of 280 nm and an emission wavelength of 303 nm, corresponding to the maximum of gibberellic acid photoproducts. Each solution was analyzed in triplicate, and a blank solution prepared identically but containing no gibberellic acid was also measured in parallel.

The proposed method was validated by using a kinetic spectrophotometric method to monitor the conversion of gibberellic acid into gibberellenic acid.[9] The initial reaction rate varied linearly for at least 2 min in 3.75 mol L^{-1} hydrochloric acid. The reaction was monitored by measuring the solution absorbance at 254 nm at 20 s intervals for 2 min. The slope of the conversion line was proportional to the initial concentration of gibberellic acid in the sample.

Procedure for Commercial Technical Formulations. Gibberellic acid in the commercial phytosanitary product Clemencuaje 1.6% was determined by dissolving 0.5 g of the sample in 50 mL of ethanol and placing appropriate volumes of the solution in 10.0 mL volumetric flasks to obtain final concentrations of the compound falling within the linear range of the calibration curve. Then, each solution was supplied with 0.8 mL of buffer at pH 5, 5 mL of methanol, and enough ethanol to obtain a final content of 5% v/v in the alcohol. The mixtures were diluted to volume with water and irradiated for 6 min prior to measuring their fluorescence intensity at 303 nm with excitation at 280 nm. The concentration of gibberellic acid in each sample was determined from the calibration curve.

Procedure for Fruit and Vegetable Samples. A previously weighed amount of each type of sample was chopped, blended, and ground to obtain a homogeneous mixture. Then, 10 g of each homogeneous sample was transferred into a 100 mL conical flask and spiked with appropriate known amounts of gibberellic acid in ethanol to ensure that the final concentration would fall within the linear calibration range after the whole extraction procedure. A volume of 25 mL of ethanol was then added to each flask prior to sonication in an ultrasonic bath for 10 min. Next, the mixtures were passed through Albet DP 125 quantitative analysis filter paper and appropriate volumes of the ethanolic extracts were placed in 10.0 mL volumetric

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flasks and diluted to a final concentration falling in the linear portion of the calibration curve prior to UV irradiation for 6 min and fluorescence measurements as described above.

RESULTS AND DISCUSSION

Factors Affecting the Fluorescence Intensity. Gibberellic acid exhibits weak native fluorescence; however, preliminary studies have shown that this plant growth regulator is converted into a fluorescent compound by UV irradiation in a methanol−water solution.

One plausible explanation for the fluorescent properties of irradiated gibberellic acid is the formation of aromatic photoproducts. A tentative reaction mechanism has been proposed by which a mixture of photoproducts exhibiting ring photoaromatization is obtained (Figure 1).[25,26] Although the experimental conditions were those where gibberellic acid fluoresces differently from those typically used in reported work, the fluorescence might be generated via a similar mechanism. It should be noted, however, that further spectroscopy study is required to identify the resulting products.

The influence of chemical and instrumental variables affecting the photochemically induced fluorescence of gibberellic acid was studied with a view to maximizing the sensitivity of its determination. The influence of the solvent was examined first. Because gibberellic acid is insoluble in water, it required prior dissolution in ethanol to prepare the stock solutions. On the basis of the effect of this solvent on the photochemically induced fluorescence spectrum of the analyte, a proportion of alcohol of 5% was adequate for the intended purpose. The presence of methanol is known to enhance the photochemically induced fluorescence emission of some herbicides.[27] This led us to examine the influence of the methanol content of the medium, using a 5% (v/v) proportion of ethanol and an irradiation time of 3.3 min. The fluorescence intensity increased markedly with incrasing content of methanol. The hydroxyl radical, ·OH, is known to be a strong oxidant with a very high standard potential. This makes alcohols useful as scavengers for it.[28,29] Although 100% methanol led to the highest fluorescence signal, the determination required aqueous samples, so an aqueous buffer was added to adjust the pH. No shift in the excitation or emission spectrum was observed by the effect of changing the polarity of the solvent. For these reasons, we chose to use such a high proportion of methanol such as 50% (v/v) for the determination.

The influence of the sample irradiation time on the fluorescence intensity was also examined. The results are shown in Figure 2. An irradiation time of 6 min, obtained by using a peristaltic pump speed of 5 rpm, was chosen because longer and shorter times led to a much lower fluorescence intensity at the emission maximum for gibberellic acid photoproducts (λ_{em} = 303 nm); thus, short irradiation times resulted in low conversion into fluorescent products, whereas long times decreased the fluorescence through subsequent reaction of the photoproducts.

The influence of pH on the fluorescence intensity was studied by adding variable amounts of HCl or NaOH to gibberellic acid solutions before irradiation. The pH range studied was 1.0−12.0. The fluorescence intensity of gibberellic acid photoproducts was nearly constant at pH 4−7. pH 5 was thus selected for subsequent use and adjusted by adding sodium acetate−acetic acid buffer to the solution. The fluorescence intensity of gibberellic acid photoproducts was not affected by the buffer concentration, however, so a 0.04 mol L^{-1} concentration was used to obtain an adequate buffering capacity.

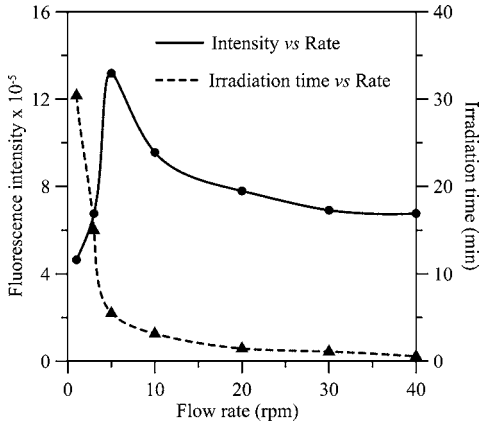

Figure 2. Influence of UV irradiation time and related flow rate on the fluorescence signal of gibberellic acid measured at 303 nm (λ_{ex} = 280 nm) in a 5% v/v ethanol and 50% v/v methanol solution.

The effect of the temperature on the fluorescence intensity was also examined. Such an effect is of great theoretical significance and practical benefit. Increasing the temperature increases molecular thermal motion, thereby also increasing the probability of radiationless transitions and reducing the fluorescence quantum yield as a result. The influence of temperature was studied over the range 15−45 °C. As expected, the fluorescence intensity decreased as the temperature was raised. A temperature of 20 °C was selected for the photochemically induced fluorescence determination of gibberellic acid.

Other instrumental parameters were optimized in order to establish the best possible conditions for the analytical determination, which included a high intensity and signal-to-noise ratio for maximal sensitivity. The optimum values of such variables were a slit width of 6.0 nm, an integration time of 0.1 s, and four averaged scans per spectrum.

Three-dimensional spectra allow the most accurate characterization of the fluorescence of a compound. Figure 3 shows the isometric projection and contour plot of the three-dimensional spectrum for irradiated gibberellic acid under the previously selected conditions. Since this isometric representation may be less useful to expose hidden emission peaks, we used the two-dimensional fluorescence contour plot for gibberellic acid photoproducts instead. The photoproducts exhibited two broad bands at λ_{ex} = 280 nm (λ_{em} = 303 nm) and λ_{ex} = 315 nm (λ_{em} = 420 nm), the former of which was the stronger, and hence, that was selected to determine the plant growth regulator.

Calibration and Analytical Parameters. The above-described optimum chemical and instrumental conditions were used to develop a method for determining gibberellic acid by measuring its photochemically induced fluorescence signal development at an emission wavelength of 303 nm and an excitation wavelength of 280 nm. The fluorescence intensity was found to increase with increasing concentrations of the analyte up to 500 ng mL^{-1}. Fitting the results revealed that the gibberellic acid concentration was linearly related to the fluorescence intensity over the range 50−150 ng mL^{-1} (Figure 4). All measurements were made in triplicate.

The proposed method was validated by statistical analysis; for this purpose, the experimental data were fitted by least-

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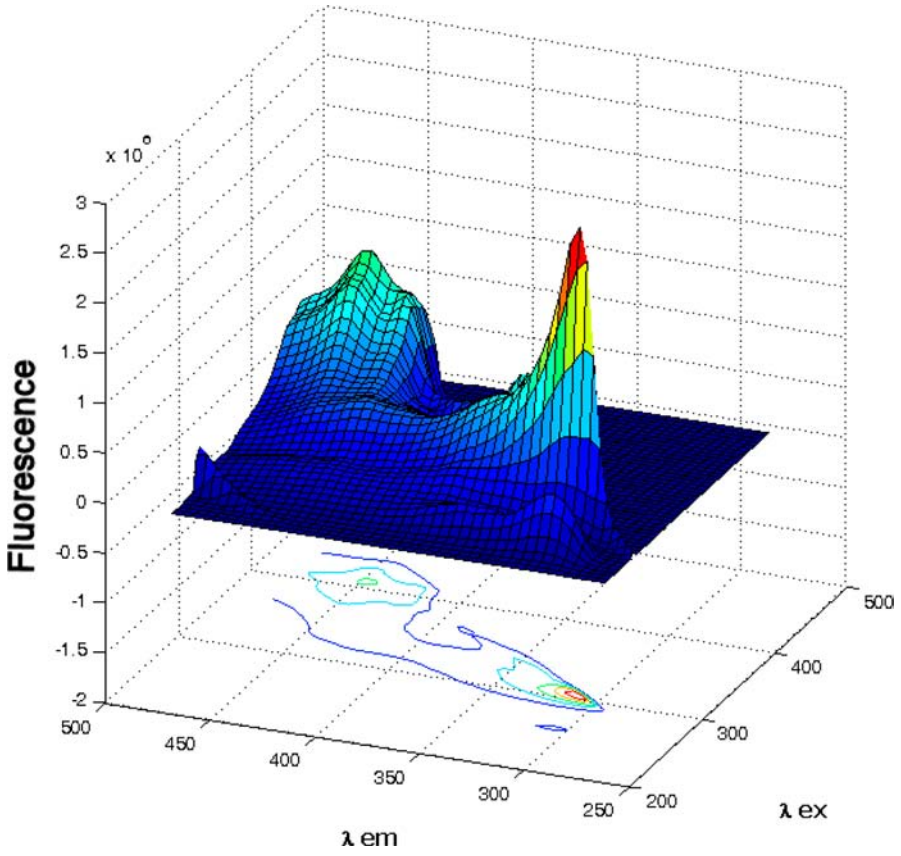

Figure 3. Total fluorescence spectrum of gibberellic acid (150 ng mL^{-1}) irradiated during 6 min in a 50% v/v methanol, 5% v/v ethanol, and pH 5 buffer solution.

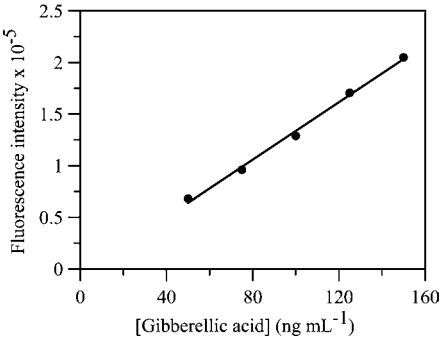

Figure 4. Calibration curve obtained for gibberellic acid.

squares regression to a linear equation: $y = a + bx$. Table 1 shows the statistical figures of merit of the determination. The high regression coefficient obtained testifies to the good linearity of the calibration curve throughout the usable analyte concentration range and the negligible scatter among experimental points.

Table 1. Figures of Merit for the PIF Determination of Gibberellic Acid in 50% v/v Methanol, 5% v/v Ethanol, and pH 5 Buffer Solution

concentration range (ng mL^{-1})	50−150
intercept on the *y*-axis	8.797×10^3
standard deviation of intercept	2.5×10^3
slope	1.268×10^3
standard deviation of slope	2.8×10^1
standard deviation of the regression	6.1×10^3
determination coefficient (r^2)	0.991

On the basis of the random residual distribution obtained, the calibration curve was homoscedastic (i.e., the variance was constant and concentration-independent throughout the dynamic range); as a result, the fluorescence intensity was linearly related to the gibberellic acid concentration throughout the concentration range studied. The statistical analysis was supplemented with the determination of the limit of detection (LOD) for the proposed method. Conventional methods for this purpose aim at preventing false positives, i.e., type I errors (reporting an analyte as present when it is not). One favored alternative is to define detection limits in such a way that both false positive and false negatives (i.e., type II errors, which are made when an analyte is reported to be absent when in fact it is present) are avoided. Our detection limit as calculated according to Clayton et al.[30] with provision for type I and II errors was 11.6 ng mL^{-1} ($\alpha = \beta = 0.005$; $n = 18$). Using the error propagation approach, which provides limits consistent with the reliability of blank measurements and signal measurements of standards,[31,32] led to a limit of 6.2 ng mL^{-1}. Finally, the limit calculated according IUPAC[32,33] was 1.7 ng mL^{-1}.

The precision of the proposed method was assessed by preparing 10 replicates of a standard solution containing a gibberellic acid concentration of 75.0 ng mL^{-1} and measuring them as described above. Using the theory of error propagation and a 95% confidence level interval for the predicted concentration with provision for variations in sample signal and standard deviations of the intercept and slope of calibration

curve led to a relative standard error of 1.9% and a relative standard deviation of 1.8%.

The determination of gibberellic acid was validated by least-squares regression.[34] The performance of the proposed method was compared with that of an existing spectrophotometric method[9] by analyzing 7 samples containing the analyte at levels within the application range. The concentrations, obtained with the spectrophotometric method and the proposed method, were subjected to least-squares paired analysis, which considers the effects of various types of error. The presence of random of errors in the test method causes points to scatter around the least-squares line and the calculated slope and intercept to slightly depart from unity and zero, respectively. The random error can be estimated from the standard deviation in the y direction (also called the standard deviation of the estimate of y on x). A proportional systematic error leads to a change in b, so the difference between b and unity provides an estimate of the proportional error. A constant systematic error reflects in a nonzero intercept. If both methods provide identical concentrations for the same samples, then the least-squares analysis gives a zero intercept and a unit slope. Figure 5 shows

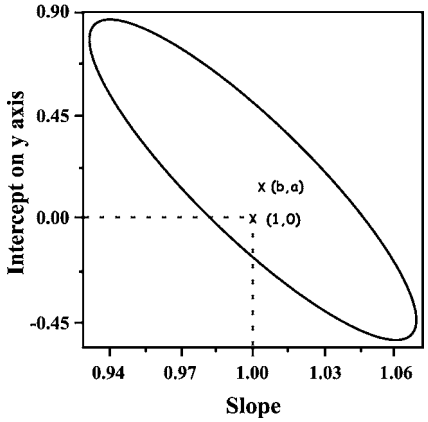

Figure 5. Comparison between the proposed method and the spectrophotometric method. The ellipse is the 95% confidence region for the true slope and the intercept on the ordinate estimated from the overall least-squares regression between the concentration calculated in reverse through both methods. The point (1,0) corresponds to the zero intercept and unit slope.

the 95% confidence region for the true slope and estimated intercept. As can be seen, the point corresponding to the zero intercept and unity slope falls within the joint confidence region, which means that the accuracy of the proposed method and the currently endorsed, spectrophotometric method is not significantly different.

The sensitivity of the proposed method affords the determination of gibberellic acid at concentrations below its MRL in all types of matrices. Our optimized method is more sensitive than existing alternatives. Thus, the spectrophotometric method of Berríos et al.[9] has a sensitivity limit of 0.1 g L^{-1} and a linear concentration range of 0.1−1 g L^{-1}, both of which are much higher than those of the proposed method. Sequential injection analysis with potentiometric detection[11] also has a higher linear range (5×10^{-4}−8×10^{-3} mol L^{-1}). Although the linear range of an existing spectrofluorimetric method for gibberellic acid[10] is broader, its LOD is somewhat higher (5 ng mL^{-1}) and similar to that of the liquid chromatography−tandem mass spectrometry method of Hou et al.,[5] which, however, uses sophisticated, expensive equipment unaffordable to most laboratories.

Applications. The proposed method was applied to the determination of gibberellic acid in a commercial phytosanitary formulation (*Clemencuaje*) containing a 1.6% proportion of the analyte. Samples were prepared as described under Experimental Section. The recoveries obtained with the proposed method were quite consistent with the nominal content of gibberellic acid in the product (Table 2).

Table 2. Recovery Study of Gibberellic Acid in Tomato and Fruit Samples by PIF

samples	added (μg g^{-1})	found (μg g^{-1})	recovery ± SD[a] (%)
tomato	6.0	5.7	95.3 ± 1.2
mandarin orange	6.0	5.9	99.2 ± 0.4
plum	6.0	5.8	96.3 ± 0.7
strawberry tree berry	6.0	5.9	98.5 ± 1.3
Clemencuaje	120.0 ng mL^{-1}	130.1 ng mL^{-1}	108.4 ± 0.3

[a]Standard deviation for $n = 3$.

The method was also used to determine gibberellic acid in Spanish organic agricultural products including tomato and fruits (mandarin orange, plum, and strawberry tree berry). Strawberry tree is a common name for *Arbutus unedo*, a tree bearing edible fruits that is native to Western Europe and the Mediterranean basin but cultivated in other temperate areas. The fruit is a red berry with a rough surface that is edible and ferments on the tree if left to ripen. Strawberry tree berries are used to make jams, beverages, and liqueurs.

The plum and strawberry tree berry samples contained no gibberellic acid because they were subjected to no chemical treatment. Also, the mandarin oranges were found to be gibberellic acid-free in preliminary tests. A recovery study was thus conducted by using standard solutions of the plant growth regulator to spike all samples in order to validate the proposed method.

Potential interferences were sought by examining the fluorescence of the samples. As can be seen in Figures 6a and b, respectively, plums and tomatoes exhibited fluorescence without irradiation; however, irradiating them under the conditions of maximum fluorescence emission by the analyte suppressed their fluorescence. As a result, the fluorescence signal obtained when the analyte was extracted from these two types of samples after irradiation was similar to that obtained from a gibberellic acid standard solution. By contrast, mandarin oranges and strawberry tree berries had no substantial natural fluorescence prior to or after irradiation.

Recovery of known amounts of gibberellic acid added to the samples (tomato, mandarin orange, plum, and strawberry tree berry) was assessed from fluorescence intensity versus the concentration calibration curve. Table 2 shows the results as average percent recoveries ($n = 3$); as can be seen, the amounts of analyte found were quite consistent with those added.

In conclusion, the proposed PIF method makes it easier and less expensive to determine gibberellic acid in foods such as tomato and fruits without the need for labor-intensive sample pretreatment. Vegetable samples such as tomato and plum contain many organic substances; some confer these foods a high background fluorescence that interferes with the direct determination of gibberellic acid. However, the strong background signals of these matrices are suppressed by UV

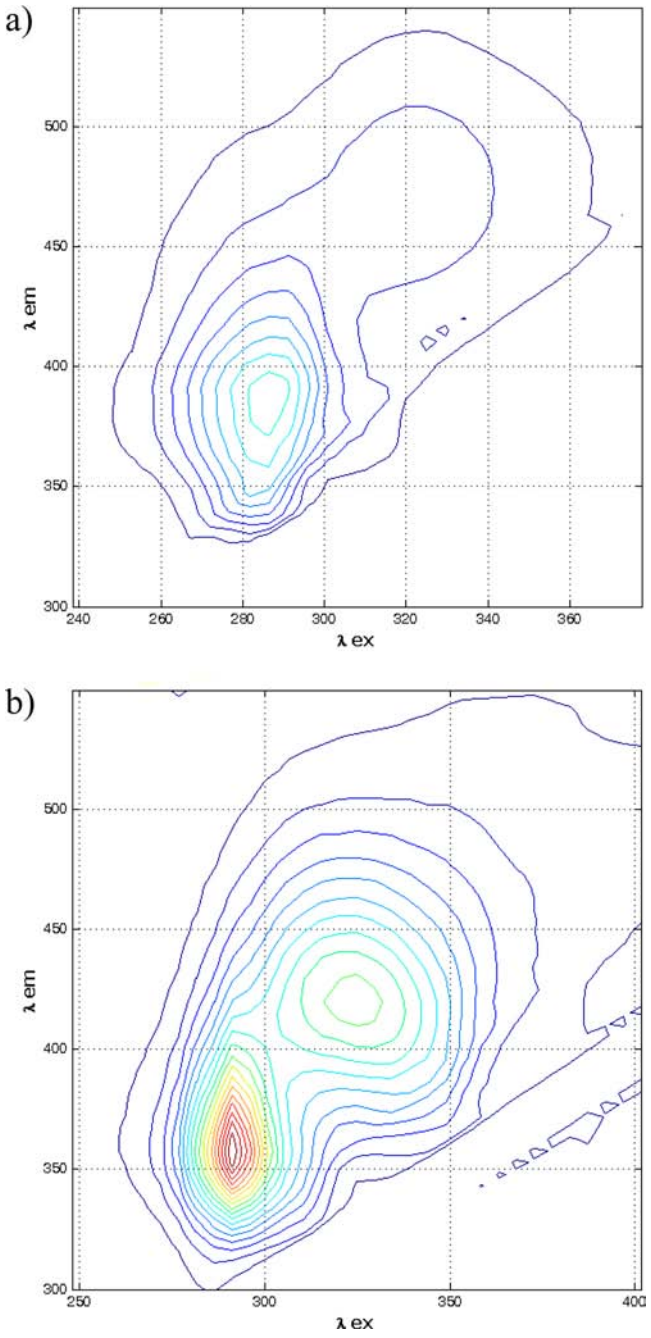

Figure 6. Total fluorescence spectra of plum (a) and tomato (b) extracts without irradiation in a 50% v/v methanol, 5% v/v ethanol, and pH 5 buffer solution.

irradiation, which additionally enhances the fluorescence of the analyte. The proposed method can be applied to a wide variety of vegetable samples with a nonfluorescence matrix before and after UV irradiation such as mandarin orange and strawberry tree berry, which testifies to its robustness. Application to agricultural products provides high analyte recoveries with no interference from the matrix. Finally, the proposed method uses simple, inexpensive instrumentation and requires no labor-intensive sample preparation.

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Notes

The authors declare no competing financial interest.

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