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Multicommuted fluorescence based optosensor for the screening of bitertanol residues in banana samples

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Abstract

A multicommuted flow through optosensor is developed in order to determine bitertanol in banana samples, by measuring the pesticide native fluorescence at 261/326 nm. The solid support used in the flow-through cell, C_{18}, allows both high selectivity and sensitivity necessary for the screening of the pesticide at residue levels. These characteristics of optosensing are implemented with low sample consumption and automation intrinsic properties of multicommutation, providing a useful tool for pesticide residue routine determination in foods. An acetonitrile extraction/partitioning and dispersive solid-phase extraction clean-up procedure proved to be useful; an additional cleaning step with C_{18} solid phase extraction (SPE) cartridges was carried out, eliminating all matrix limitations. Recovery experiments performed on banana samples at different concentrations provided recoveries between 81% and 115%. A detection limit of 0.014 mg kg^{-1} was obtained. This makes the method suitable for screening bitertanol in banana samples, fulfilling the maximum residue levels of 3 mg kg^{-1} established by the European Union.

Keywords: Optosensing; Multicommutation; Bitertanol; Banana

1. Introduction

Bitertanol (BIT) (β-(1,1′-biphenyl)-4-yloxy-α-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol) (Fig. 1A) is a systemic azole fungicide, widely used for the protection of several crops, mainly for control of *Sigatoka* in banana plantations (Zamora, Hidalgo, López, & Hernández, 2004). Due to the possible harmful effects of pesticides, a strict legislative framework is established in order to control the maximum amount of pesticides permitted in food. The maximum residue level (MRL) for each pesticide is established both by the European Union and the Spanish government. In the case of bitertanol residues in banana samples, the MRL established is 3 mg kg^{-1} (www.mapya. es).

The determination of pesticides at residue levels in vegetables is a difficult task, due to the complexity of the matrix along with the low concentration levels at which the analytes are usually found, as well as the possible interferences due to the very complex matrix. Therefore a highly sensitive technique is required, together with an extraction and an additional sample clean-up steps in order to remove the possible interferences in the analysis.

Usually, bitertanol determination in vegetables is carried out using liquid chromatography (Blasco, Font, & Picó, 2002; Juan-García, Mañes, Font, & Picó, 2004; Zamora, Hidalgo, et al., 2004; Zamora, Pozo, López, & Hernández, 2004) or gas chromatography (Gelsomino, Petrovičová, Tiburtini, Magnani, & Felici, 1997; Sicbaldi, Sarra, Mutti, & Bo, 1997; Štajnbaher & Zupančič-Kralj, 2003) techniques. An extraction process is required in order to separate the analyte from the matrix; typically an organic solvent or a mixture of solvents is used in the extraction process. After that, a suitable dilution and filtration (Zamora, Hidalgo, et al., 2004, 2004), solid phase extraction (SPE) step (Juan-García et al., 2004; Štajnbaher

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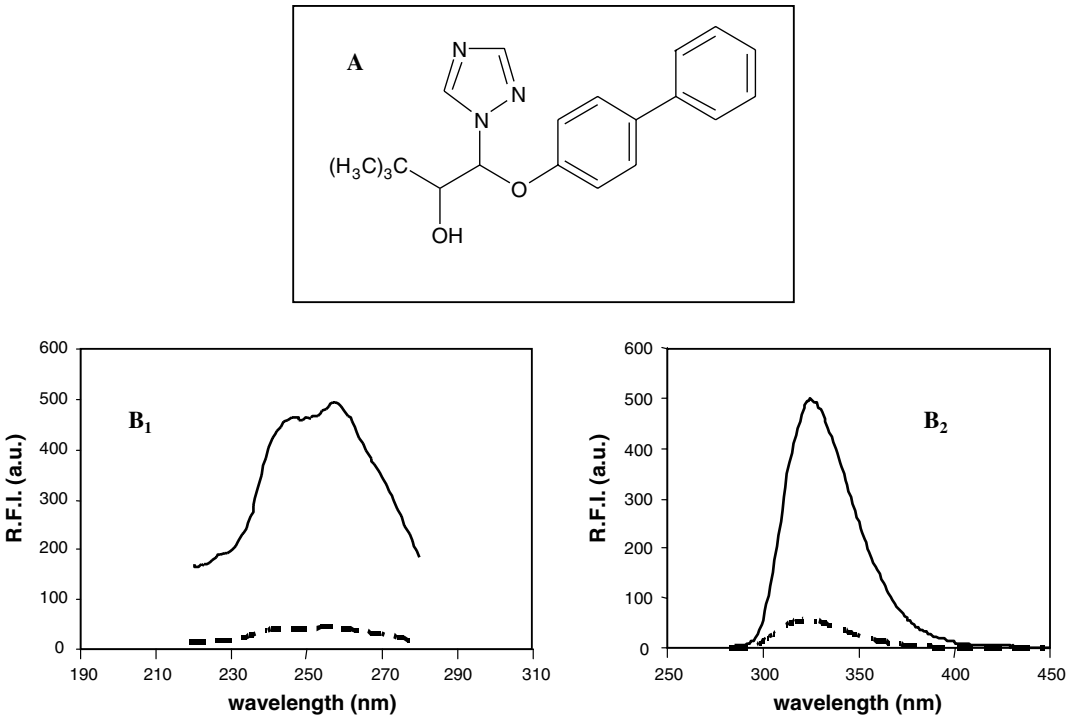


Fig. 1. (A) Chemical structure of bitertanol. (B) Excitation (B_1) and emission (B_2) Fluorescence spectra of bitertanol (100 ng/ml) retained on the solid support (C_{18} silica gel) (solid line) and in aqueous solution (dashed line).

& Zupančič-Kralj, 2003), gel permeation chromatography (Gelsomino et al., 1997) or stir bar sorptive extraction (Blasco et al., 2002; Juan-García et al., 2004) is required before injection into the chromatograph.

Multicommutation refers to the use of solenoid valves controlled automatically by using appropriate software; the solenoid valves are individually switched on and off by means of an electric pulse. When compared to typical flow injection analysis (FIA), better repeatability, lower sample and reagent consumption, easier sample handling and minimal waste generation are observed as main advantages (Catalá Icardo, García Mateo, & Martínez Calatayud, 2002; Rocha et al., 2002).

Flow-through optosensing (Fernandez-Sanchez, Segura Carretero, Cruces-Blanco, & Fernandez-Gutierrez, 2003; García-Reyes, Llorent-Martínez, Ortega-Barrales, & Molina-Díaz, 2004a) provides higher sensitivity when compared with typical flow injection analysis (FIA); in addition it provides the selectivity needed when analysing complex samples. For instance, the analysis of pesticides in natural waters has been studied using optosensing (García-Reyes et al., 2004a, García-Reyes, Llorent-Martínez, Ortega-Barrales, & Molina-Díaz, 2004b). Coupling multicommutation to flow-through optosensing maintains the advantages of both methodologies, providing a highly effective analytical method (Llorent-Martinez, Dominguez-Vidal, Ortega-Barrales, de la Guardia, & Molina-Diaz, 2005; Llorent-Martínez, Ortega-Barrales, & Molina-Díaz, 2005).

In this paper, a multicommuted flow-through optosensor is developed in order to determine BIT by retaining it into a non-ionic solid support, C_{18}, and measuring its native fluorescence. The suitability of using an acetonitrile extraction/partitioning and dispersive SPE clean-up with primary secondary amine (PSA) has been tested. This extraction methodology is called QuEChERS, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003). The dispersive-SPE with PSA removes many polar matrix components from the food extracts, such as organic acids, pigments or sugars, eliminating potentially interfering compounds. After this clean-up, an additional step involving C_{18} cartridges had to be used in order to improve the stability of the sensing zone. Finally a recovery study was performed by spiking banana samples with BIT, obtaining satisfactory results, showing the accuracy of the developed procedure.

2. Experimental

2.1. Reagents and solutions

BIT (Riedel-de-Haën, Seelze, Germany) stock solution of 200 μg ml^{-1} was prepared in methanol. The solution was kept in dark at about 4 °C.

Methanol, acetonitrile, sodium chloride and anhydrous magnesium sulphate were obtained from Panreac (Barcelona, Spain).

Sephadex-SP C-25 in sodium form, 40–120 μm average particle size (Sigma–Aldrich, Buchs, Switzerland), Sephadex G-15, 40–120 μm average particle size (Sigma–Aldrich) and C_{18} bonded phase silica gel beads (Waters, Milford, USA) with 55–105 μm of average particle size, were tested as solid supports.

In the extraction procedure, Supelclean PSA SPE (primary secondary amine, solid phase extraction) bulk packing obtained from Supelco (Bellefonte, PA, USA) was used. AccuBond II ODS-C18 cartridges (500 mg solid phase; 6 ml volume) from Agilent Technologies (Palo Alto, CA, USA) were used in the clean-up step.

2.2. Instrumentation

Luminescence measurements were performed with a Cary-Eclipse Luminescence Spectrometer (Varian Inc., Mulgrave, Australia). The spectrometer was connected to a computer with a Cary-Eclipse (Varian) software package for data collection and treatment.

A Hellma flow cell 176.752-QS (Hellma, Mülheim, Germany) (25 μl of inner volume and a light path length of 1.5 mm) was used. The cell was filled with C_{18} solid phase microbeads, and was blocked at the outlet with glass wool to prevent displacement of the support particles.

The manifold is illustrated in Fig. 2A. A four-channel Gilson Minipuls-3 (Villiers Le Bel, France) peristaltic pump with rate selector and methanol-resistant pump tubes type Solvflex (Elkay Products, Shrewsbury, MA, USA) were used. An electronic interface based on ULN 2803 integrate circuit (Motorola, Phoenix, AZ, USA) was employed to generate the electric potential (12 V) and current (100 mA) required to control the four 161T031 NResearch three-way solenoid valves (Neptune Research, West Caldwell, NJ, USA). The software for controlling the system was written in Java. Flow lines of 0.8 mm internal diameter PTFE tubing and methacrylate connections were used.

The level of the solid support packed in the flow cell had to be carefully selected, in order to ensure that the light beam passes completely through the solid layer. Higher levels would imply that the support zone where the species of interest is sorbed would fall outside the detection area and so, a lower and wider signal would be obtained; with lower levels, the light beam would pass through the solution completely or partially and, consequently, a decrease in the signal would be obtained. So, the top of the solid support is kept as close as possible to the light beam, this latter being completely covered by the support. The selected level involved the use of 40 mg of C_{18} silica gel in the flow cell. In order to condition the solid support, before starting the measuring process the carrier solution was passed through the sensing zone for 5 min.

2.3. Sample extraction and clean-up

Banana samples were obtained from the local markets. "Blank" extracts were used to prepare matrix-matched standards for optimisation purposes. One kilogram of the fruit sample was chopped and homogenised with a high-speed blender (Taurus, Oliana, Spain). A representative 15-g portion of sample was weighed in a 50 ml PTFE centrifuge tube. 15 ml of acetonitrile was added and the tube was vigorously shaken for 1 min; 2.5 g of NaCl and 6 g of anhydrous $MgSO_4$were then added and the tube was vigorously shaken again for 1 min. After this time, it was centrifuged at 3700 rpm for 3 min. 5 ml of the supernatant (acetonitrile phase) were taken with a pipette and transferred to a 15 ml centrifuge tube; 250 mg of PSA and 750 mg of $MgSO_4$ were added. The tube was vigorously shaken for 20 s and centrifuged again (3700 rpm) for 3 min. Thus, an acetonitrile extract containing 1 g of sample per ml was obtained.

2.5 ml was taken and diluted 10-fold with deionised water. A clean-up extraction procedure with reverse-phase C_{18} cartridges (500 mg solid phase; 6 ml volume) was carried out. The retained BIT was then eluted using 2.5 ml of MeOH and diluted 5-fold with deionised water. This solution was used as the sample inserted in the flow manifold.

To perform the recovery studies, a representative 50 g portion of a blank banana sample previously homogenised was weighted and transferred to a glass mortar, where it was fortified homogenously with about 3 ml of a bitertanol methanol standard solution of an appropriate concentration. The mixture was then gently blended in the mortar to assess the homogeneity of the sample. The sample was then allowed to stand at room temperature for 8 h, before it was kept in the fridge until analysis.

2.4. General procedure

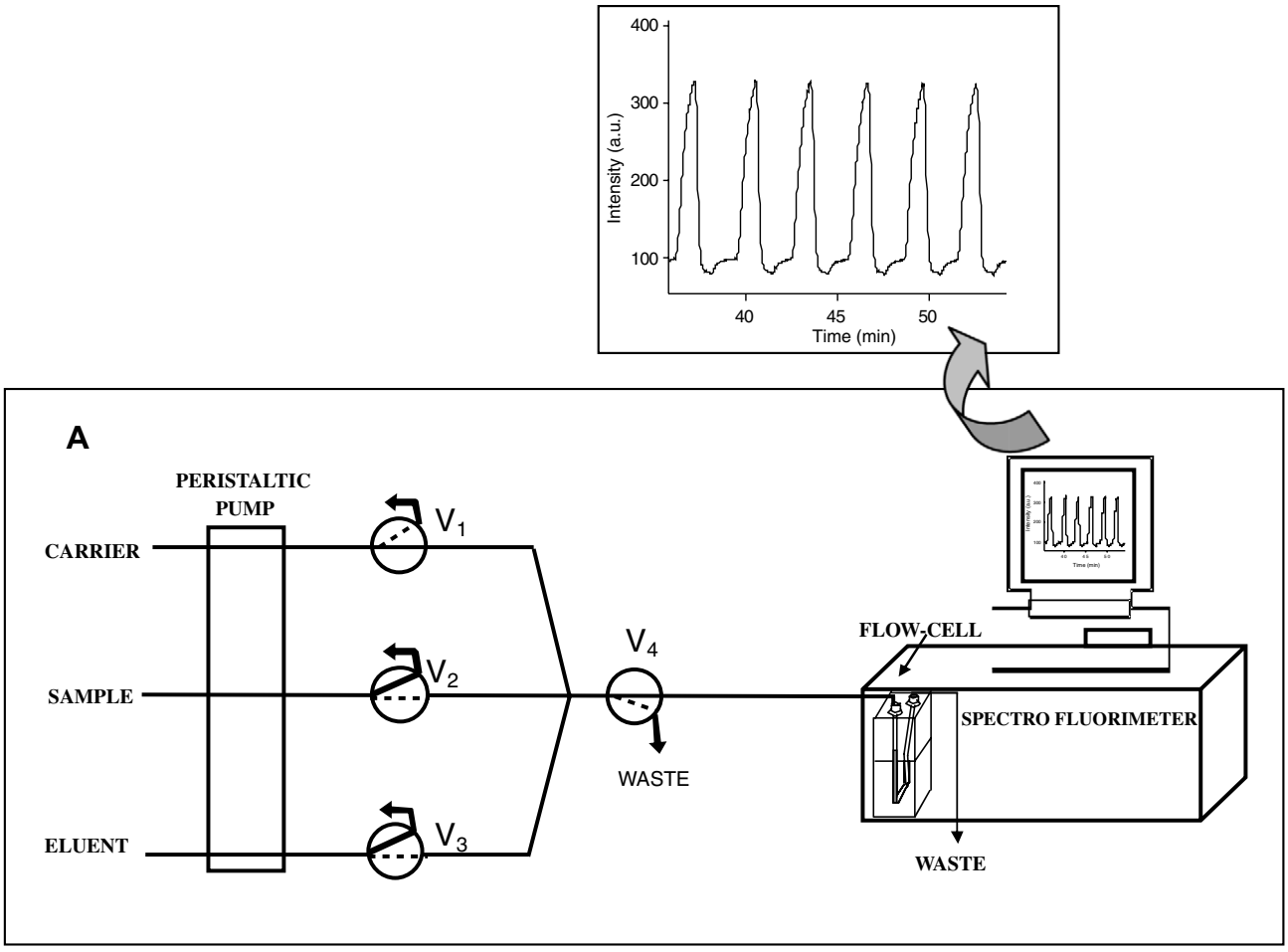
The flow network procedure is shown in Fig. 2B. In the initial status, all valves are switched off and the carrier, 70% MeOH, is flowing through the flow-through cell while all other solutions are recycling to their vessels. The sample is introduced by simultaneously switching on valves $V_1$and V_2 for 60 s (1 ml sample) (step 1; Fig. 2B). BIT is carried towards the flow-through cell where it develops its analytical signal, being eluted from the solid support by the carrier itself (step 2 in Fig. 2B). After BIT is eluted, by switching valves V_1 and V_3 on for 60 s, MeOH is introduced into the flowing system in order to completely regenerate the solid phase. Finally a tubing cleaning step comprising two steps: step 4a: 5 s introduction of the following sample diverted to waste (by means of V_4); step 4b: 5 s of carrier diverted to waste (by means of V_4) is carried out in order to avoid contamination between samples.

Calibration standards and samples were analyzed in triplicate. Luminescence measurements were made at excitation/emission wavelengths of 261/326 nm, respectively. A typical signal recording can be observed in Fig. 2C.

3. Results and discussion

3.1. Selection of the solid support

The selection of the solid support was performed taking into account the structure of BIT (Fig. 1); it has a basic pK of 3.63 and an acidic pK of 13.74. So it can be present



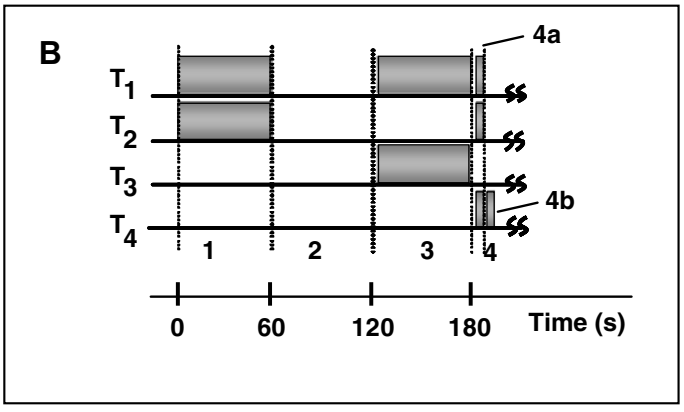


Fig. 2. (A) Flow manifold. In each valve, the straight line indicates the flow direction when the valve is off, and the dotted line the direction of the liquid when the valve is switched on. (B) Valves scheme. T_1, T_2, T_3 and T_4 refer to the timing courses of solenoid valves V_1, V_2, V_3 and V_4. The shadow surface above the valves timing course line indicates that the corresponding valve was switched on. The steps were the following. 1: sample introduction (60 s); 2: BIT-transitory signal and elution (60 s); 3: C_{18} cleaning step with MeOH (60 s); 4: tubing cleaning step (step 4a: sample introduction diverted to waste (5 s); step 4b: carrier introduction diverted to waste (5 s)). (C) Profile of the transient signal showing the signal obtained for a BIT concentration of 0.4 mg kg^{-1}. The baseline shift is observed at different methanol concentration (depends on the compacting of the solid support in each medium). When the elution of BIT finishes, there is a slight decrease in baseline due elution with 100% MeOH, and the increase of the baseline (signal increase before the BIT peak) is due to the 70% MeOH carrier.

either in cationic, non-charged or anionic form depending on the pH. Sephadex SPC-25 was tested as cationic resin. G-15 and C_{18} silica gel was tested as non-ionic solid supports. Anionic solid supports were not tested due to the very high pK_a which would make necessary the use of a very basic medium.

For pH lower than 3, BIT is present mainly as cationic species, while for higher pH it is non-ionic. For acidic pH the retention of BIT in SPC-25 was observed, but the signal was lower than that obtained when using non-ionic supports at an appropriate pH (higher than 5). C_{18} provided the highest signal, and the elution was easily achieved by means of MeOH, so it was chosen as solid support.

3.2. Instrumental variables

The maximum excitation and emission wavelengths of BIT in aqueous solution and retained on the C_{18} solid support were 258/325 nm and 261/326 nm, respectively. The later values were chosen for the development of the method. The fluorescence spectra are shown in Fig. 1 (B_1 and B_2).

The instrument excitation and emission slit widths were established as 5 and 20 nm, respectively, in order to obtain the optimum value for the analyte signal/C_{18} background signal. The voltage of the photomultiplier tube was set at 600 V as a compromise between sensitivity and signal-to-background ratio had to be achieved.

3.3. Sample treatment

3.3.1. QuEChERS

The acetonitrile extraction used in this paper has been previously described (Anastassiades et al., 2003). This extraction provided good recoveries, so it was not necessary to make any modifications.

3.3.2. C_{18} cartridge clean-up

After the acetonitrile extract was obtained, 5- and 10-fold dilutions with deionised water were tested with direct insertion into the flow system without any further treatment, but a signal decrease was observed between injections. This fact could be attributed to some matrix components which may be retained on the solid support. The regeneration of the solid support could not be achieved only with MeOH, so an additional sample clean-up step was necessary.

We included a clean-up step using C-18 as sorbent material in order to remove matrix components which may be retained on the C-18 solid phase in the final determination step. As every interfering substance was removed in this clean-up step, no decrease in the signal was observed any more between injections.

For this clean-up step, the acetonitrile extract was diluted 10-fold in order to provide conditions where bitertanol was quantitatively retained on the sorbent. The solution was passed through the cartridges and finally BIT was eluted with 2.5 ml of MeOH. Then, 5- and 10-fold dilutions were inserted in the flow system; 5-fold dilution provided better signals than the 10-fold dilution, so it was selected for further studies.

3.4. Chemical variables

The chemical variables studied were the nature of carrier and eluting solutions. These studies were undertaken using matrix-matched standards.

Taking into account the non-polar nature of the C_{18} silica gel support, the carrier and eluting solutions were prepared in different proportions of methanol and water. The optimisation of the solutions was carried out with the optimum amount of the solid support in the flow-through cell (40 mg C_{18}).

The carrier solution was tested by using aqueous solutions with different MeOH percentages, up to 80% (v:v). The signal diminished when increasing the MeOH percentage, but for solutions with less than 70% MeOH BIT was not eluted from the solid support. So 70% was chosen as optimum. In order to completely regenerate the baseline 100% MeOH was used after each injection.

3.5. Flow variables

The sample introduction time and the flow-rate of the peristaltic pump were the studied variables. The sample introduction time was studied ranging from 10 to 180 s. When increasing the sampling time, the signal increased due to a higher amount of analyte being introduced into the flow system, so the amount of analyte sorbed on the solid support was higher. If the sample was prepared in water, the signal increased up to 180 s, but if the sample was prepared in a banana blank extract, the signal only increased up to 60 s, being this one the selected insertion time. This difference in preconcentration process could be attributed to the effect of the banana matrix.

The flow rate was studied from 0.6 up to 1.2 ml min^{-1}. When increasing the flow-rate the signal also increased, due to a higher amount of sample being introduced into the system. The frequency-rate was also increased; so the highest possible flow-rate was selected. For flow-rates higher than 1 ml min^{-1} a lower repeatability was observed, so 1 ml min^{-1} was chosen as optimum.

3.6. Figures of merit

Taking into account the optimised conditions, the analytical parameters of the system were studied using matrix-matched standards. The calibration was performed using matrix-matched standards with bitertanol concentrations in the range from 0.045 to 1 mg kg^{-1}. As it can be seen in Table 1, the system responds linearly in this concentration range. In addition, the RSD was 1.8% for a BIT concentration of 0.15 mg kg^{-1} ($n = 10$). The calculated detection and quantification limits (following the σ criteria) were 0.014 and 0.045 mg kg^{-1}, respectively. All the analytical parameters are detailed in Table 1. Taking into account the obtained parameters and the legally established MRL, the proposed methodology fulfils the requirements to

Table 1
Analytical parameters

Parameter	
Linear dynamic range (mg kg^{-1})	0.045–1
Calibration graph	
Intercept	19.4
Slope (kg mg^{-1})	496
Correlation coefficient	0.9989
Detection limit (mg kg^{-1})	0.014
Quantification limit (mg kg^{-1})	0.045
RSD (%) ($n = 10$)	1.8[a]

[a] For a concentration level of 0.15 mg kg^{-1}.

determine BIT residues in bananas. In addition, it also can be used as a screening procedure to check the content of bitertanol residues in other fruits and vegetables, where the MRLs of bitertanol are lower (0.05 mg kg^{-1} in most cases).

3.7. Interference study

The possible interference from other common pesticides was examined. A pesticide was considered to interfere if an error higher than ±5% was observed; if such an error occurred, the interferent concentration was diminished until the interference disappeared. All the obtained results are detailed in Table 2.

The interference from benomyl was not tested because it would degrade to carbendazim in the extraction procedure, so the tolerated ratio would be the same as for carbendazim.

In the case of carbofuran and carbaryl, if they are present, the tolerated ratio could be drastically increased taking into account their interaction with the C_{18} solid phase; carbofuran and carbaryl require 40% and 55% MeOH, respectively in order to be eluted from C_{18} solid phase, as previously reported (García-Reyes et al., 2004a; Llorent-Martinez, Garcia-Reyes, Ortega-Barrales, & Molina-Diaz, 2005). As BIT needs 70% MeOH as described in the proposed methodology, two strategies could be used in order to avoid the interference:

- By changing the procedure in the C_{18} cartridges clean-up step: instead of using directly 100% MeOH for the elution of BIT, 50% MeOH could be first used. Both interferents would be eliminated. After that, bitertanol is eluted from the SPE cartridge with 100% MeOH.

Table 2
Study of interferences from pesticides

Pesticide	Tolerated interferent/analyte (w/w) ratio[a]
Imazalil, aminocarb, bendiocarb, simazine, aldicarb, dimethoate	>20[b]
Carbendazim, phenitrothion	15
Carbofuran	4
Carbaryl	1

[a] For a 0.4 mg kg^{-1} BIT concentration.
[b] Maximum ratio tested.

Table 3
Recovery study

Sample	Spiked (mg kg^{-1})	Recovery (%)	RSD (%)
Banana-1	0.05	111	1.3
	0.2	94.7	0.7
	0.5	81.5	1.1
Banana-2	0.2	95	2.4
	0.4	99.9	1.5
	0.6	114.9	1.8
Banana-3	0.1	109.5	1.4
	0.2	98	2.3
	0.4	100.3	1.2
Banana-4	0.05	91.2	2.5
	0.3	108.4	1.3
	0.5	98.3	1.7

- If the clean-up step with the cartridges is not changed, a precolumn filled with C_{18} could be used in the system (although the valves configuration should be slightly changed). 50% MeOH would be used in order to eliminate the interfering compounds, and then 70% MeOH as carrier.

3.8. Analytical applications

Following the general procedure previously described, the determination of BIT in banana samples was carried out. Taking into account the MRL, 3 mg kg^{-1}, banana samples were spiked with BIT concentrations below this threshold in order to assess the feasibility of residue determinations using the proposed methodology.

To perform the recovery studies, fortified banana samples were prepared as describe in Section 2.3. In all the studied samples, recoveries values between 88% and 111% were obtained, as it can be observed in detail in Table 3.

4. Conclusions

The suitability of applying a multicommuted fluorescence based optosensor to the analysis of pesticide residues in complex matrixes such as fruit samples is here demonstrated. The determination of BIT is satisfactorily achieved by means of an acetonitrile extraction and solid phase extraction clean-up with PSA procedure, followed by an additional clean-up step with C_{18} cartridges. The marriage between multicommutation and native fluorescence based optosensing provides a synergy between both methodologies advantages, such as high sensitivity and selectivity, as well as speed and low sample and reagents consumption, requirements for these kinds of samples. The method fulfils the MRL levels established for BIT, making it suitable for screening analysis of this pesticide in banana samples, as was demonstrated with recovery experiments.

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