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Rapid Determination of Diphenylamine Residues in Apples and Pears with a Single Multicommuted Fluorometric Optosensor

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In this work, a single flow injection multicommuted system using solid-surface fluorescence spectroscopy has been explored for the determination of diphenylamine in apples and pears. The native fluorescence signal of diphenylamine retained on the solid support (C_{18} silica gel) was used for its determination ($\lambda_{exc}/\lambda_{em}$ 291/372 nm). The sample treatment consists of a liquid−liquid extraction with acetonitrile followed by a cleanup step using primary−secondary amines. The use of a continuous flow assembly implementing multicommutation, based on a set of three-way solenoid valves controlled by appropriate software, provides the automatic control of sample, carrier, and eluting solution with remarkable advantages in relation to conventional flow injection approaches. Using an optimized sampling time, the proposed method was linear in the range 0.25−5 mg kg^{-1} with a detection limit of 0.06 mg kg^{-1} and RSD (percent) values better than 3%. The proposed method was successfully applied to the analysis of diphenylamine in different apple and pear samples fortified at different concentrations, and recoveries between 78 and 104% were found. The results obtained illustrate the usefulness of the proposed method for the screening and evaluation of postharvest treatment of crops possibly containing diphenylamine.

KEYWORDS: Diphenylamine; pesticide; optosensor; multicommutation; food analysis

INTRODUCTION

Postharvest treatment of crops with fungicides is a worldwide agricultural practice used to control postharvest decay caused by various fungal pathogens. Because of this, residues of fungicides are frequently found in agricultural commodities. In this sense, long-term exposure of apples and pears to low temperatures in controlled-atmosphere storage commonly induces a physiological disorder known as scald (*1*). Diphenylamine (DPA) is an agrochemical product widely employed to control storage scald on apples and pears. It is applied mainly to help control apple and pear scald and to prolong their storability without deterioration. Formulations used for this purpose, include an emulsifiable concentrate, a wettable powder, a soluble concentrate/liquid, and a ready-to-use liquid. DPA is applied by dipping, drenching, or spraying (*1*, *2*).

This usage and the possible negative effects of DPA and its derivatives in human health have prompted the establishment of maximum residue limits (MRLs) in commodities on which DPA is used. According to EU regulations in foodstuffs, the use of DPA in pears and apples is approved with relatively high tolerance levels (5 and 10 mg kg^{-1} for apples and pears, respectively) (*3*).

The determination of DPA residues in fruits is a difficult task, mainly because of the complexity of the matrix. It usually requires a dedicated sample treatment procedure with various extraction and cleanup stages. The determination of DPA in crops has been undertaken mainly by chromatographic methods. First, gas chromatography with nitrogen−phophorus was applied (*4*). Gas chromatography−mass spectrometry with derivatization has been used for the determination of DPA in apples and pears (*5*). Reversed-phase high-performace liquid chromatography with fluorescence (*6*), electrochemical (*7*), and mass spectrometric detection (*8*) has also been employed. In general, the greater the sensitivity and selectivity of the detection technique used, the more minor are the sample treatment and cleanup steps required. In this sense, the use of liquid chromatography−mass spectrometry reduces the sample treatment steps because of its inherent selectivity and sensitivity (*9*).

In this work, a new method for the determination of DPA in apples and pears has been developed. The proposed methodology is based on native fluorescence measurements carried out on a solid support [in this case, a nonpolar sorbent (C_{18} silica gel)] aiming to increase the selectivity of spectroscopic measurements in relation to conventional fluorescence spectroscopy (*10*, *11*). This detection principle (solid phase optosensing at active surfaces) has been implemented in a multicommuted continuous flow assembly (*12*, *13*), which consists of a set of three-ways solenoid valves, which permits an automated and

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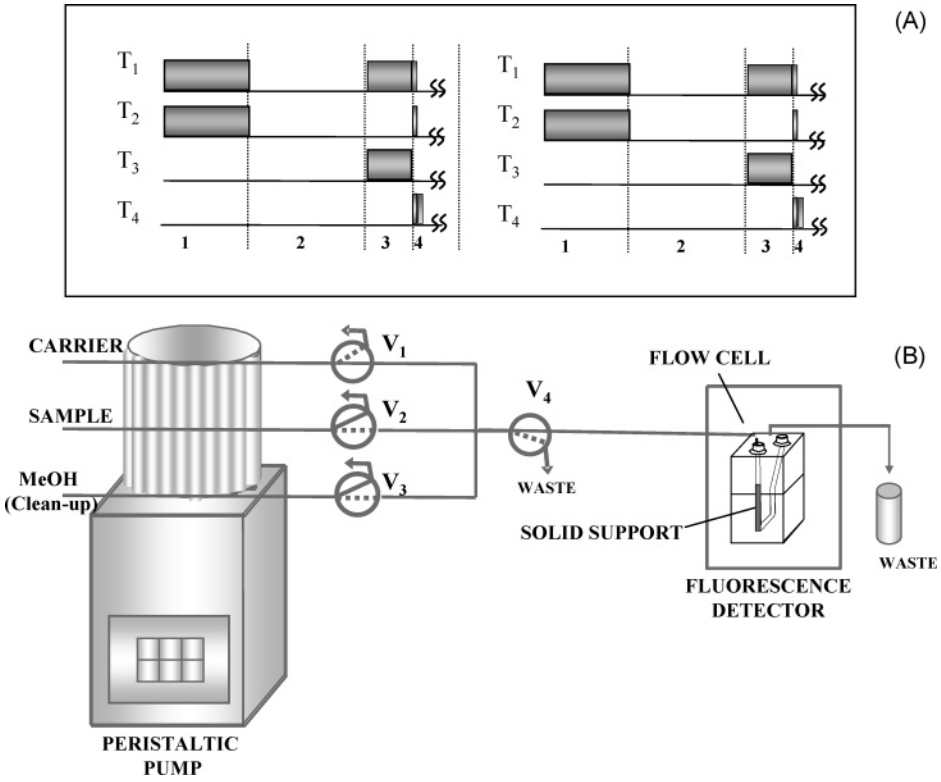

Figure 1. (**A**) Valve scheme. T_1, T_2, T_3, and T_4 refer to the timing courses of solenoid valves V_1, V_2, V_3, and V_4, respectively. The shaded surface above the valve timing course line indicates that the corresponding valve was switched on. The flow procedure comprised the following steps: (1) sample introduction; (2) diphenylamine transitory signal and elution; (3) solid support cleaning step with MeOH; (4) tubing cleaning step. (**B**) Flow manifold.

programmable control of sample and reagent aliquots, reducing remarkably the volume of sample, reagents, and waste, in relation to conventional flow injection systems. This flow assembly is controlled by a computer with appropriate software to design multiple flow schedules.

Therefore, the advantages of solid-phase optosensing concept are complemented with those of multicommuted flow systems in order to develop a single and fast method for the determination of DPA in apples and pears. These kinds of systems exploiting these two principles have been called multicommuted flow-through optosensors (*14*, *15*). Separation and partial matrix removal, preconcentration, and detection steps are performed automatically and simultaneously without sample manipulation during the measurements. This automated methodology has been combined with a single sample treatment procedure to extract DPA. It involves a liquid−liquid partitioning step followed by a cleanup stage based on solid-phase dispersive extraction using primary−secondary amines (PSA) as sorbent (*16*).

The developed method is single, sensitive (meets the European Union MRL regulations of DPA residues in both pears and apples), and cost-effective. Therefore, it can be used for monitoring DPA residues in real samples and also for the evaluation of postharvest treatment of apples.

EXPERIMENTAL PROCEDURES

Reagents and Materials. All standard solutions both for optimization studies and for calibration were prepared from analytical reagent grade chemicals by using doubly distilled water. Diphenylamine (Riedel-de-Haën) stock solution of 500 μg cm^{-3} was prepared by dissolution of 50 mg in 100 cm^{-3} of methanol (Merck, Darmstadt, Germany). The solution was kept away from light at ~4 °C. Working solutions were prepared by suitable dilution of the stock solution in 20% MeOH aqueous solutions. Methanol, acetonitrile, sodium chloride, and magnesium sulfate anhydrous were obtained from Panreac. Supelclean PSA-SPE (Supelco) (PSA, solid-phase extraction) bulk packing was used in the cleanup stage of the extraction procedure. C_{18} bonded phase silica gel beads (Waters, Milford, MA) with 55−105 μm average particle size was used as solid support.

Apparatus and Instruments. The multicommuted continuous-flow assembly used is outlined in **Figure 1**. It was built using a four-channel Gilson Minipuls-3 (Villiers Le Bel, France) peristaltic pump with a rate selector (the same flow rate for each channel), and methanol-resistant pump tubes of type Solvflex (Elkay Products, Shrewsbury, MA) were used. An electronic interface based on a ULN 2803 integrated circuit was employed to generate the electric potential (12V) and current (100 mA) required to control the four 161T031 NResearch three-way solenoid valves (Neptune Research, West Palm Beach, FL). The software for system control was developed in Java. Flow lines of 0.8 mm internal diameter poly(tetrafluoroethylene) (PTFE) tubing and methacrylate connections were also used.

Fluorescence measurements were performed with a Varian Cary-Eclipse fluorescence spectrofluorometer (Varian Inc., Mulgrave, Australia), equipped with a Hellma (Jamaica, NY) 176.052-QS quartz flow-through cell (25 μL of inner volume and a light path length of 1.5 mm). The spectrofluorometer was connected to a computer with a Cary Eclipse software package for data collection. The flow cell was filled with C_{18} silica gel beads, as a slurry suspension in methanol, with the aid of a syringe. The flow-through cell was blocked at the outlet with glass wool, to avoid displacements of the C_{18} gel beads.

Sample Treatment. A multiresidue extraction procedure involving an acetonitrile extraction/partitioning and dispersive solid-phase extraction (SPE) cleanup with PSA was used to extract DPA from both pears and apples (*16*). The dispersive SPE with PSA removes many polar matrix components from the food extracts, such as organic acids, pigments, or sugars, removing potential interference compounds.

Pear and apple samples were obtained from local markets. "Blank" extracts (in DPA) (checked by HPLC-MS) were used to prepare the matrix-matched standards for validation purposes. One kilogram of the fruit sample was chopped and homogenized with a high-speed blender. A representative 15 g portion of this sample was weighed in a 50 cm^3 PTFE centrifuge tube; 15 cm^3 of acetonitrile was added, the tube was

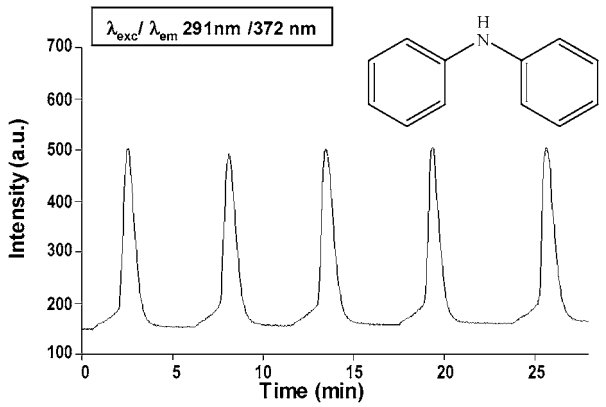

Figure 2. Profile of typical transient signals obtained in a pear extract spiked with 3 mg kg^{-1} of diphenylamine ($n = 5$).

vigorously shaken for 1 min; 1.5 g of NaCl and 6 g of anhydrous $MgSO_4$ were then added; and the tube was vigorously shaken again for 1 min, preventing the formation of $MgSO_4$ conglomerates. After this time, it was centrifuged at 3700 rpm for 3 min; 5 cm^3 of the supernatant (acetonitrile phase) was taken with a pipet and transferred to a 15 cm^3 centrifuge tube; 250 mg of PSA and 750 mg of anhydrous $MgSO_4$ were added. The tube was energetically shaken for 20 s and centrifuged again (3700 rpm) for 3 min. Finally, an extract containing 1 g of sample per cubic centimeter in 100% acetonitrile was obtained. Before analysis by the proposed method, the acetonitrile fruit extract was diluted 50-fold with 20% MeOH.

General Procedure. In the initial status, all valves are switched off and the carrier, 50% MeOH, flows through the flow-through cell (1.10 mL min^{-1}) while all other solutions are recycling to their vessels. The sample is introduced by simultaneously switching valves V_1 and V_2 for 80 s. DPA is carried toward the flow-through cell, where it develops its analytical transitory signal (fluorescence measurements were made at excitation/emission wavelengths of 291/372 nm) being first preconcentrated in the sensing phase and subsequently eluted by the carrier itself. Then, MeOH is introduced into the flowing system by switching valves V_1 and V_3 for 40 s. This step is performed to eliminate possible matrix residues in the solid support, avoiding thus deactivation of the solid support, rendering it ready for another sample injection. The flow network procedure is shown in **Figure 1**. As an example, a diagram with the recorded signals for DPA in a spiked pear extract is shown in **Figure 2**.

RESULTS AND DISCUSSION

Preliminary Studies. *Spectral Features and Instrumental Variables.* The spectral features of DPA were recorded at room temperature (25 °C) in both aqueous solution and gel-phase media. The effect of pH on the fluorecence intensity was not critical; thus, no buffer was required. Fluorescence spectra, which were collected in working aqueous solution, showed maximum excitation/emission wavelengths at 281/394 nm. The spectra of DPA fixed on C_{18} silica gel beads showed maximum excitation/emission wavelengths at 291/372 nm. The change of the fluorescence spectrum can be attributed to the modification of the surrounding environment of the analyte in the solid phase with regard to that on solution. The selected excitation and emission wavelengths to carry out the determination of DPA were 291/372. Relative fluorescence intensity measurements performed in gel-phase media are usually affected by background signal levels higher than those found in homogeneous solution, obviously owing to the presence of the solid support in the irradiated zone. For this reason, instrumental parameters were carefully investigated to achieve the best possible signal-to-background ratio. A study of the photomultiplier tube voltage in the range of 400−800 V was also carried out. As a compromise between sensitivity and background signal due to the solid support, the photomultiplier tube voltage was set at 700 V. The instrument excitation and emission slits widths were studied in the range from 1 to 20 nm width. Widths of 5 and 20 nm were established for excitation and emission slit widths, respectively.

Selection of Solid Support. Taking into account the structure of DPA [see **Figure 2** (inset)], the more appropriate retention behavior of the analyte on the solid support was adsorption mechanism. Two solid supports were tested: Sephadex G-15 (dextran-type gel without exchangeable groups) and a nonpolar sorbent (C_{18} silica gel). From the results obtained, C_{18} bonded silica gel beads proved to be the most suitable sensing material because they provided a higher signal. Moreover, the regeneration step and stripping of possible matrix residues after several sample injections can be easily accomplished with methanol when using C_{18} silica gel as solid support, in the same way as it is usually performed in reversed-phase HPLC.

Chemical Variables. The chemical variables studied were the nature of carrier and eluting solutions. The extract of the sample was diluted 50-fold with an aqueous solution of 20% MeOH prior to analysis, to avoid possible damage of the active surface of the solid support by insoluble matrix particles. The high sensitivity of the proposed procedure makes possible this previous dilution.

Taking into account the nonpolar nature of C_{18} silica gel beads, several carrier solutions were prepared containing methanol and water in different proportions, for the appropriate preconcentration and subsequent elution of DPA. Optimization of the carrier solution was carried out with methanol solutions with proportions in the range from 40 to 60% MeOH (v/v). The methanol percentage did not affect strongly the analytical signal, although the sampling frequency and the total time of analysis varied considerably. Moreover, carrier solutions with methanol percentages lower than 45% did not elute completely the analyte, obtaining peaks with large tails. Finally, a 50% MeOH (v/v) solution was the optimum value for a complete desorption, high signal peak, and low signal time.

On the other hand, although the baseline was completely regenerated, in preliminary repeatability studies, we observed that after some (5−10) sample insertions, the signal decreased by ∼2−5% for each injection. This was probably due to some components of the matrix being retained on the solid support. To avoid this, a cleanup and regeneration step with 100% MeOH (40 s) was added to the flow schedule. This step provides the removal of these components from the matrix, rendering the solid support ready for the next injection, and also increased the lifetime of the sensor.

Study of Flow Variables. An interesting feature of solid-phase spectroscopic detection is the potential to improve the sensitivity by increasing the flow variables: both the flow rate and time introduction signal can modulate the sensitivity of the developed method, because the higher the flow rate, the higher the analyte mass injected and, therefore, the higher the signal. This is due to the preconcentration step, which occurs in the solid support in the detection area itself (*10*). The sample introduction time and the flow rate of the peristaltic pump were thus the studied variables.

Flow Rate. The flow rate was studied from 0.50 to 1.2 mL min^{-1}, using a sample introduction time of 60 s. The higher the flow rate, the higher the amount of analyte retained and, thus, the higher the signal. On the other hand, with higher flow rates, the elution time progressively decreased while the sampling frequency increased. A 1.1 mL min^{-1} flow rate was

Table 1. Interference Study: Tolerated Ratios of Potentially Interfering Species

foreign species	tolerated interferent/analyte (w/w) ratio[a]
imazalil, imidacloprid, simazine	>50[b]
carbendazim[c]	5
O-phenylphenol[c]	4
thiabendazole[c]	2

[a] 3 mg kg^{-1} diphenylamine. [b] Maximum ratio tested. [c] Fluorophore.

chosen. Higher flow rates values could not be used as a consequence of overpressure problems in the flow system.

Sample Introduction Time. When the sampling time was increased, the signal increased due to a higher amount of analyte being introduced into the flow system, so the amount of analyte retained on the solid support was higher. It has to be taken into consideration that the presence of matrix changes the preconcentration process. In fact, the sample prepared in solvent (20% MeOH) gave a continuous increase of the analytical signal up to 220 s, whereas when the apple and pear extracts containing DPA were injected, the signal increased up to only 80 s, this being the selected insertion time.

Study of Potential Interfering Species. To evaluate the selectivity of the proposed method, a study of species that could potentially interfere in the fluorescence signal of DPA was undertaken. In this study, foreign species that are likely to be present in real samples, such as other common pesticides (some of them fluorogenic), which are widely used even in commercial pesticide formulations with DPA, were added to solutions containing DPA and their influence on the analytical signal was investigated. Tolerance level was defined as the amount of foreign species that produced an error not exceeding ±5% in the determination of the DPA. From the results obtained shown in **Table 1**, it can be concluded that DPA can be analyzed, without significant errors, in the presence of relatively high concentration levels of potentially interfering compounds.

Analytical Performance. The analytical parameters of the system were studied, under selected conditions. To confirm the suitability of the method for the analysis of real samples, matrix-matched standards were used in the calibration. With 50-fold dilution of acetonitrile extracts in 20% MeOH, the matrix effect due to fluorescence signal suppresion was negligible. In both matrices, the slope obtained was very similar to that obtained using standards in pure solvent, with ratios between the slopes obtained with matrix and solvent-based standards in the range from 0.96 to 1.03. Quantitation was carried out using peak height as analytical signal. Linearity was evaluated by analyzing different matrix-matched standard solutions at different concentration levels in the range from 0.25 to 5 mg kg^{-1}. Taking into account the 50-fold dilution, the concentration range of injected solutions is 5−100 μg L^{-1}. As can be observed in **Table 2**, the linearity of the analytical response across the studied range is excellent, with correlation coefficients better than 0.999. The remarkable sensitivity of the method can be attributed to solid-phase spectroscopic measurements, which provides better analytical features in terms of sensitivity and selectivity when compared to conventional fluorometric measurements performed in liquid phase (*10*, *11*).

The repeatability of the method was also evaluated on matrix-matched solutions at two different concentration levels. The repeatability study was carried out by injection of the same standard solution ($n = 10$). The RSD values obtained from run-to-run studies are summarized in **Table 2**. From the results

Table 2. Analytical Parameters of the Proposed Method for the Determination of Diphenylamine in Apples and Pears

parameter	apple	pear
linear dynamic range/mg kg^{-1}	0.25–5	0.25–5
calibration graph		
intercept	1.92	0.96
slope/kg mg^{-1}	131.3	126.3
correlation coefficient	0.9991	0.9998
detection limit/mg kg^{-1}	0.06	0.06
quantification limit/mg kg^{-1}	0.2	0.2
RSD (%) ($n = 10$)	1.5[a]	1.8[a]
	2.4[b]	2.2[b]

[a] Concentration level = 3 mg kg^{-1}. [b] Concentration level = 1 mg kg^{-1}.

Table 3. Recovery Studies of Diphenylamine in Apples and Pears

sample	spiked (mg kg^{-1})	recovery (%)	RSD[a] (%)
apple 1	1	88.3	2.8
	1.5	91.0	3.0
	2	86.8	1.9
apple 2	1	92.3	2.2
	2	89.7	2.5
	3	96.1	1.7
apple 3	1	104.0	1.8
	2	86.8	2.0
	4	91.2	1.4
pear 1	1	91.1	2.6
	1.5	85.7	2.9
	2	88.0	2.1
pear 2	1	87.1	1.8
	2	92.9	2.8
	3	87.0	2.0
pear 3	1	78.3	2.4
	2	89.0	2.1
	4	87.1	1.6

[a] $n = 3$.

obtained, the developed method was found to be precise (with run-to-run instrumental RSD values below 2%). On the other hand, the detection limits (LODs) were estimated from the injection of matrix-matched standard solutions at concentration levels corresponding to a signal-to-noise ratio of ~3. The results obtained in both matrices are included in **Table 1**. In general, the proposed method meets the requirements regarding the MRLs imposed by existing regulations in Europe (*3*). Moreover, in terms of sensitivity and LODs, the proposed optosensor compares well against other previously reported methods for DPA based on either GC or HPLC (*4*−*8*). On the other hand, it provides remarkable advantages in terms of speed of analysis, cost-effectiveness, and simplicity, representing an alternative for the screening of DPA residues in both apple and pear extracts.

The method was applied to the determination of DPA residues in apples and pears sample purchased from different markets. However, the DPA content of all the samples was smaller than the above-stated detection limit. Therefore, to evaluate the accuracy of the whole method (extraction and determination), different recovery studies were accomplished by spiking both apple and pear matrices at different concentration levels, being then extracted and analyzed with the developed method. A representative 50 g portion of a blank sample, previously homogenized, was weighed and transferred to a glass mortar, where it was fortified homogeneously with 5 mL of a DPA 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 before it was kept in the refrigerator until analysis. The recovery percentages obtained, between 78 and 104%, shown in **Table 3**, illustrate the usefulness of the developed method, which can be applied to determine DPA residues in both apples and pears.

Conclusions. A method for the determination of diphenylamine residues in pears and apples has been developed in this work. The proposed automated method is based on native fluorescence measurements of DPA on a solid support (C_{18} silica gel), after a single sample treatment step based on a liquid−liquid partition step followed by a "cleanup" based on a solid-phase dispersive extraction step. The use of multicommutation allows the automated control of sample and reagent aliquots, with remarkable savings of sample, reagent, and waste (in relation to classic FIA systems). The method is single, cost-effective, and sensitive enough to meet European Union regulations regarding MRLs of DPA in apples and pears. The proposed method could be used to monitor and evaluate postharvest treatment of these crops.

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