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STUDY OF AGRICULTURAL SAMPLES BY LASER DESORPTION COUPLED WITH RESONANCE-ENHANCED MULTIPHOTON IONIZATION AND TIME-OF-FLIGHT MASS SPECTROMETRY: APPLICATION TO CARBENDAZIM ANALYSIS IN PEPPER

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A method for the analysis of carbendazim in peper has been developed using Supercritical Fluid Extraction (SFE) for the sample preparation and the combination of Laser Desorption (LD) with Resonance Enhanced Multi-Photon Ionization (REMPI) coupled to Time-of-Flight Mass Spectrometry (TOF-MS) for the detection. The method allows the analysis of carbendazim in pepper reducing the sample preparation step and avoiding the matrix effects present in classical GC and HPLC techniques, as well as the problems related to the labile properties of carbendazim. The detection limit obtained is of the same order of magnitude as the generally employed HPLC technique, but with higher sensitivity and easier sample preparation.

Keywords: Laser mass-spectrometry; multiphoton ionisation; carbendazim; laser desorption

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1. INTRODUCTION

Carbendazim (methyl benzimidazol-2-ylcarbamate) is a systemic fungicide with protective and curative action, effective for control of a wide range of fungal diseases in cereals, fruits, vegetables and other crops [1]. It is used in many countries throughout the world and its residues are commonly found by regulatory agencies in pesticide residue monitoring and total diet studies [2–4]. Benomyl (another benzimidazol-type fungicide) and thiophanate-methyl (a benzimidazole-precursor-type fungicide) degrade in plants to give carbendazim. Both benomyl and thiophanate-methyl rapidly convert into carbendazim during the extraction procedures commonly used to determine their residues in crops [5]. For these reasons, Maximum Residue Limits (MRLs) corresponding to these three fungicides are established as a sum of benomyl, carbendazim and thiophanate-methyl expressed as carbendazim. Some examples of the MRLs established by the Spanish and/or the European Union legislations for these pesticides are: 5.00 mg/kg in citrus fruits, 2.00 mg/kg in peppers and 0.50 mg/kg in melons [6].

Due to the extensive use of these pesticides, it is of great interest to have a suitable analytical method to determine its residues in agricultural samples [7]. Carbamate pesticides have been mainly analysed by gas chromatography (GC) [8, 9] and high performance liquid chromatography, (HPLC) [10–12]. Since carbendazim is a labile compound, the technique usually employed for the analysis of carbendazim is reversed phase HPLC with fluorescence or ultraviolet detection [13, 14]; this technique requires a long procedure for the separation and extraction of the compound prior to its analysis [10, 15, 16]. The detection limit of the conventional method for the analysis of benzimidazoles adapted to carbendazim is 0.02 mg/kg for 5 mg samples and 0.01 mg/kg for 8 mg samples [17].

The recent development of analytical-scale supercritical fluid extraction (SFE) has opened new perspectives to improve the sample preparation step in any analytical process [18]. SFE has advantages of increased automation, greater selectivity, reduced sample preparation time, lower operating costs, and much less waste generated, versus the traditional extraction methods requiring organic solvents. In addition, when carbon dioxide is used as the extraction solvent, concentration of the analytes after extraction in SFE is convenient and fast because

supercritical CO_2 becomes a gas after depressurization. Also, CO_2 is safe, unreactive, readily available, relatively inexpensive, and has a low critical pressure and temperature point, making it, for all these reasons, the most common solvent in SFE [19].

From the late 1980's, a variety of applications of SFE have been developed involving environmental contaminants, fats and oils, natural products and others [20, 21], but the inherent advantages in SFE have been scarcely exploited in the analysis of pesticide residues in fruits and vegetables as this technique presents some practical limitations to be applied to high-water content samples [22]. Recently, some authors have demonstrated that the best way to solve these limitations is to mix fruits and vegetable samples, prior to SFE, with an appropriate drying agent, such as diatomaceous earth or anhydrous magnesium sulphate. The excellent results obtained by these authors indicate that SFE can be a suitable alternative to conventional extraction methods to extract different types of pesticides, including benzimidazol fungicides, from a great variety of fruits and vegetables [23–27].

On the other hand, the combination of REMPI and TOF-MS is one of the most promising analytical methods for the selective detection of trace compounds in complex matrices and solid samples [28–41]. The main advantages of this technique are the selective ionisation of minor components in a complex matrix, the great sensitivity and resolution, a major ionisation efficiency and the possibility of simultaneous analysis of different components present in the sample.

In addition, Laser Desorption is a powerful technique that produces both ions and neutrals, and has been already used to vaporise all kind of molecules, minimising the thermal fragmentation [42–48]. Coupling this technique with REMPI-TOF-MS allows the analysis of thermally unstable compounds such as carbendazim.

The present work was aimed with a view to develop a new method for the analysis of carbendazim in agricultural samples in a more direct manner, without the tedious and time consuming sample preparation necessary for classical techniques. This has been achieved by the use of Supercritical Fluid Extraction (SFE) for the sample preparation, and the combination of Laser Desorption (LD) with Resonance-Enhanced Multi-Photon Ionization (REMPI) coupled to Time-of-Flight Mass Spectrometry (TOF-MS) for the detection.

After finding the resonant wavelength of the substance and subsequent optimisation of the experimental conditions, we analysed several samples of SFE pepper extract enriched with carbendazim. The detection limit obtained using this technique is of the same order of magnitude as published for HPLC. However, the present technique shows more sensitivity and it requires less time for sample preparation than the latter. These results show the capability of our method for the determination of carbendazim in agricultural samples.

2. EXPERIMENTAL SECTION

(A) Preparation of the Pepper Extract by SFE

Reagents and Apparatus

(a) An ISCO SFE system, consisting of one Model 260 D syringe pump and controller, a SFX 2–10 extractor with restrictor heater set at 70°C, and 10 mL stainless steel extraction cartridges with removable 2 μm frits, was used in this study. Uncoated and deactivated fused silica capillary column, 30 cm length × 50 μm i.d., was used as a restrictor, and a 10 mL graduated test tube, immersed in a 15–20°C water bath, was used as collection vessel.
(b) All the solvents used were Panreac, pesticide residue grade. Carbon dioxide (99.995% purity) was supplied by SEO (Madrid, Spain). Anhydrous magnesium sulphate (> 99% purity) was obtained from Fluka.
(c) Fresh peppers were obtained from Campos de Nijar S. A. (Almería, Spain). These peppers were no treated with benzimidazol fungicides, and were determined not to contain any detectable pesticide residue by using conventional solvent-based extraction methods, and GC and HPLC analysis.

Experimental Procedure

The sample preparation and SFE methods used to obtain the pepper extract were those proposed in Refs. [25] and [26] to analyze pesticide residues in fruits and vegetables by SFE. Specifically, 20 g or blended

fresh pepper sample was thoroughly mixed with 28 g of anhydrous magnesium sulphate in a glass beaker immersed in an ice/water bath. After 5 min, the mixture was thoroughly pounded in a porcelain mortar until a dry and homogeneous powdered mixture was obtained. Extraction was carried out in a 10 mL extraction cartridge filled with 8 g of the pepper-$MgSO_4$ mixture, placing first a 1 g layer of $MgSO_4$ to bind water that migrates during extraction. Extraction was performed in a dynamic mode after a static equilibrium period of 1 min. SFE conditions were as follows: pressure, 300 atm; temperature, 50°C; pressurized CO_2 volume, 15 mL; static modifier, 200 μL methanol; collection solvent, 3 mL ethyl acetate. Pressurized CO_2 flow rate during extraction was around 1.3 mL/min. After extraction, the volume of the SFE extract (ca 1.1 mL) was adjusted to 2.2 mL with cyclohexane. This final extract contained 1.5 g of pepper sample per mL.

(B) Experimental Laser Technique

The experimental set-up is composed of two vacuum chambers designed and built in our Institute. In the first chamber, called the ionisation chamber, the following processes take place: sample desorption, ionisation of the desorbed molecules and acceleration of the ions towards the second chamber, which is the time of flight tube and where the detection of the ions is performed. Figure 1 shows a schematic view of the experimental set-up; the inset shows the internal parts of the ionization chamber, *i.e.*, accelerating plates, deflectors and ionic lens. The two chambers, connected through a gate valve, have independent vacuum systems composed of a turbomolecular and a rotary pump each a Varian V250 + Telstar RD 18 and Varian V70 + Telstar 2P-3 respectively. During the measurements the pressure in the two chambers was always kept below 10^{-6} mbar, and the second chamber was kept at pressure below 5×10^{-8} mbar when the first chamber was opened for sample manipulation.

The system for the acceleration of the ions is of the Whiley-McLaren type, composed of a set of plates each one connected to a high power supply (Stanford Research System Inc., models PS325 and PS350); the acceleration is done in two steps: V1 → V2 and

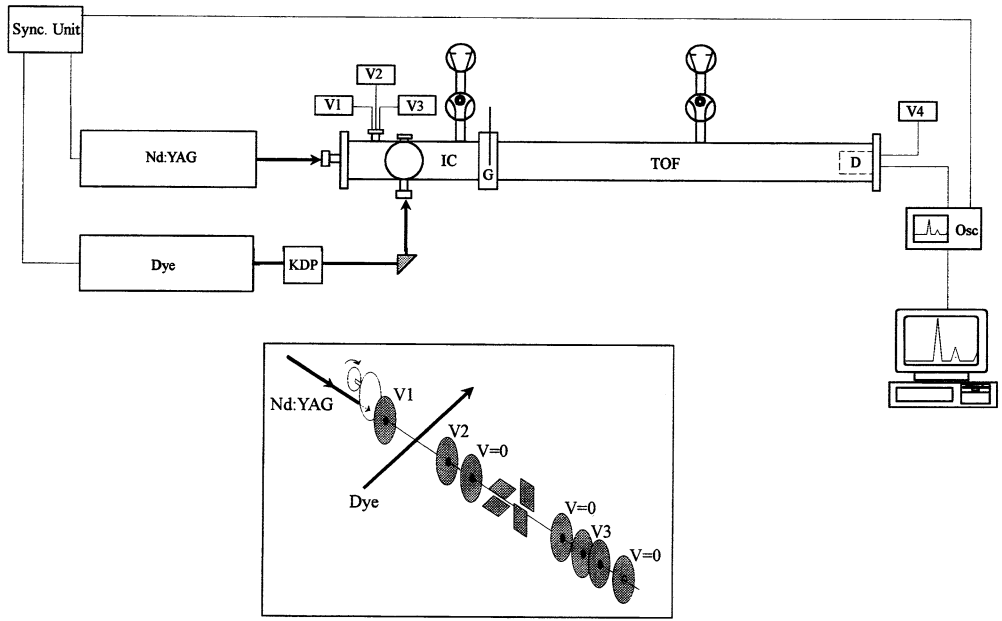

FIGURE 1 Experimental setup. The inset shows the internal parts of the ionization chamber. IC stands for the ionization chamber, TOF for the time of flight tube, V1, V2 for the accelerating voltages and V3 for ionic lens voltage.

V2 → V3 = 0. The system also includes two deflectors in the directions *Y* and *Z*, perpendicular to the TOF trajectory, and an ionic Einzel lens to keep the ions in the detector direction.

The sample is prepared dissolving the carbendazim (Aldrich, 98%) in acetone (Panreac, 99%) and then sprayed on a Pyrex disc (ϕ = 75 mm) by means of an airbrush. The disc is placed on a sample holder parallel to the first accelerating plate at 2 mm from it. The disc is rotated by a step motor during the analysis in order to have a fresh surface for the laser to desorbe. The whole system is placed on a positioner that allows the adjustment of the *Z* direction perpendicular to the desorption laser; this movement increases the number of runs for a given sample.

For the sample desorption the second harmonic of a Nd:YAG laser (Continuum Surelite) was used, with pulses of 5 ns of duration; the desorption energies ranged from 3 up to 15 mJ. The pulsed beam of desorbed molecules expands into the acceleration region and are ionized later by the 4th harmonic of a second Nd:YAG laser (Continuum ND81) or by a frequency-doubled dye laser (Continuum ND60). The ionization laser is perpendicular to the one used for the desorption and intercepts the desorbed molecules at a variable distance from the desorption zone adjustable between 6 and 30 mm.

The ions produced by the second laser pulse were accelerated towards the TOF tube by the acceleration system previously described. The ions are detected by a two microchannel plate detector (Comstock CP-625C/50F) placed at the end of the tube and the signal is collected by a digital scope (Tektronix 540) averaging 100 laser shoots. For some runs a Tektronix 11403A digital scope, with much higher resolution, was used. The TOF spectrum is then transferred to a computer for data analysis and storage.

The delay between the desorption and ionization lasers was controlled by a pulse generator (Lyons Instruments PG 75A); the lasers frequency was 10 Hz, and the pulse energies were monitored either by a pyroelectric detector (Gentec Ed-100A) for low pulse energies ranging between 0 and 10 mJ or by a calorimetric detector (Photon Control 25) for energies ranging from 10 up to 40 mJ. Variable diaphragms were incorporated in the system, to control the size of both desorption and ionization areas. For the calibration experiments in which a gas sample was used, the sample holder and

motor were replaced by a gas inlet consisting of a 6 mm diameter tube with a 0.5 mm diameter nozzle. The gas pressure was controlled in order to keep the chamber pressure below 10^{-6} mbar. The ionization laser was used only for these experiments.

3. RESULTS AND DISCUSSION

To calibrate our system in mass a first test was carried out introducing a gas mixture of toluene and aniline which were ionized using 2 mJ pulses of the 266 nm laser. The choice of toluene and aniline was based not only on their well known multiphoton ionization spectra, but also on the fact that their masses differ from one a.m.u. The test was fully satisfactory indicating a mass resolution of ca. 800 a.m.u.

Once the system was calibrated and in order to find the optimal conditions for the analysis, a pure sample of carbendazim was desorbed with the 2nd harmonic of the Nd:YAG laser and post-ionised with the 4th harmonic of the other Nd:YAG laser. The desorption energy was optimised to obtain the maximum signal which was obtained with a 8 mJ/pulse; at higher energies the signal decreases due to fragmentation of the molecule. The ionisation energy was then changed between 1 mJ and 3 mJ. Both laser beams were introduced in the chamber without focusing. Finally also the delay between both lasers was optimized in order to get the maximum carbendazim signal.

Table I summarizes the most relevant experimental conditions used in this work for the desorption and ionization of the carbendazim, as well as other relevant features. Figure 2 shows a carbendazim TOF mass spectrum obtained by multiphoton ionization at these conditions. Notice not only the excellent signal to noise ratio, but also the lack of any significant fragments yield.

To perform the resonance enhanced mass spectrometric analysis it is necessary to know the highly resolved REMPI spectrum of the compound to be analysed so as to find the optimal wavelength for selective ionisation. As described in a previous paper [49] our group has found the REMPI spectrum of carbendazim lying between 280 and 283 nm, with a significant maximum at 281.1 nm, as it is displayed in Figure 3.

TABLE I Experimental conditions

Pressure in the Ionization Chamber (mbar)	$1 \times 10^{-6} - 5 \times 10^{-7}$
Pressure in the TOF chamber (mbar)	$1 \times 10^{-7} - 5 \times 10^{-8}$
V_1 (volts)	1840
V_2 (volts)	1580
V_3 (volts)	230
V_{MCP} (volts)	2000
Ionization to desorption distance (mm)	7,5
Laser Repetition rate (Hz)	10
Laser pulse duration (ns)	4–6
Delay between lasers (μs)	25
λ desorption (nm)	532
E desorption (mJ/pulse)	10
λ ionization (nm)	281.1
E ionization (μJ/pulse)	150–550
Number of shots/measurement	100

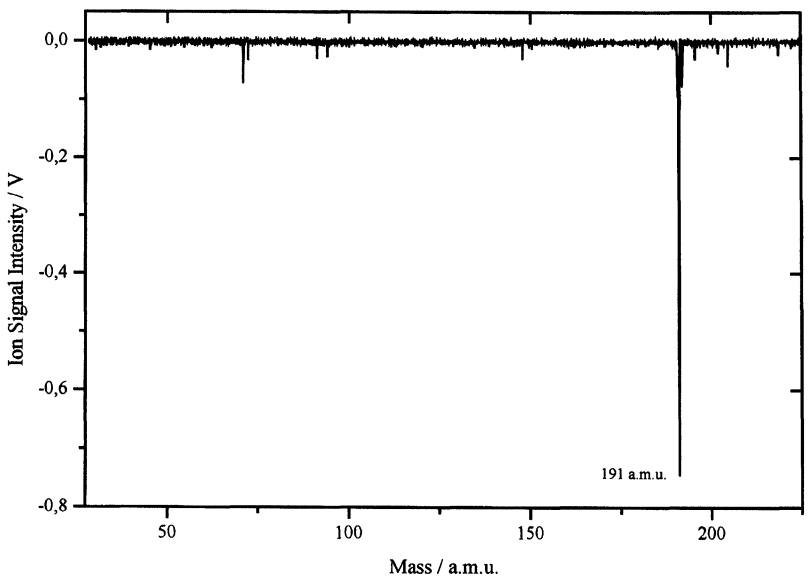

FIGURE 2 Carbendazim TOF mass spectrum.

The samples for the analysis of carbendazim were prepared dissolving the compound in a pepper extract obtained by SFE. The extract was fortified with several amounts of carbendazim to determine both the detection limit and the sensitivity of the technique. Between two consecutive analysis, both vacuum chambers were heated

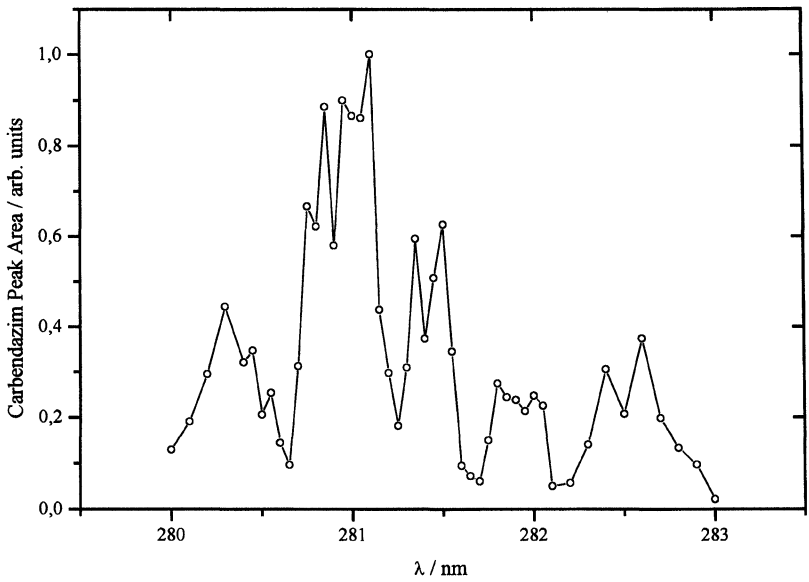

FIGURE 3 REMPI spectrum of carbendazim.

overnight, but in fact a two hours heating period proved to be sufficient to avoid any residue coming from the previous sample. A liquid nitrogen trap, currently under design, will be placed below the desorption zone to strongly reduce the background and so the heating period between consecutive runs.

Prior to the analysis of carbendazim, we studied a pure pepper extract sample in the same conditions as the final analysis to verify whether there was some peak at 191 a.m.u. that could interfere with our results. Figure 4 shows the TOF mass spectrum obtained from the blank, which was prepared by spraying 20 mL onto the disc. The absence of any significant signal at 191 a.m.u. is noticeable. With regards to the question, what does the pepper extract consist of, no full analysis was carried out of this extract. We shall emphasize that the main objective of the present investigation is focussed on the analysis of carbendazim, regardless of other components present in the analyte. As stated above, the combination of selective ionization plus the versatility of the time-of-flight spectrometry, allows one to clearly identify and analyze one component without inferences due to the rest

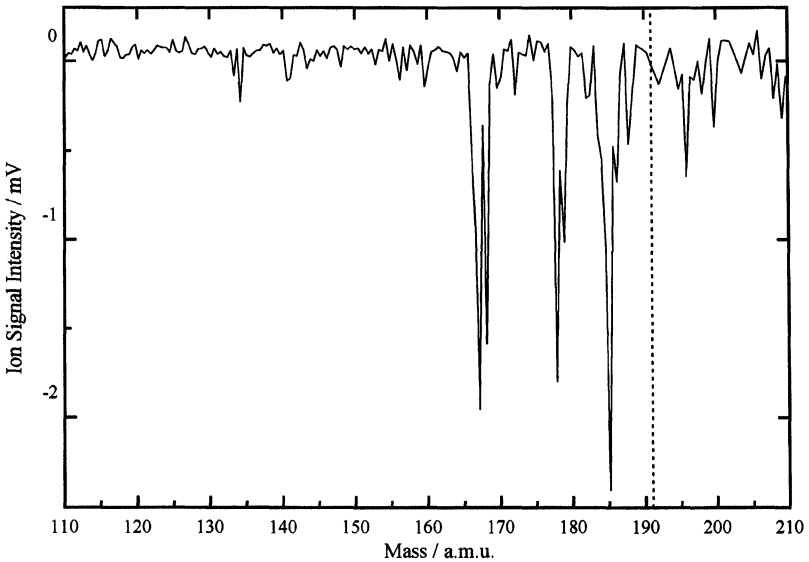

FIGURE 4 TOF mass spectrum of the pepper extract in the region of the carbendazim signal. (λ_d = 532 nm, λ_i = 281.1 nm).

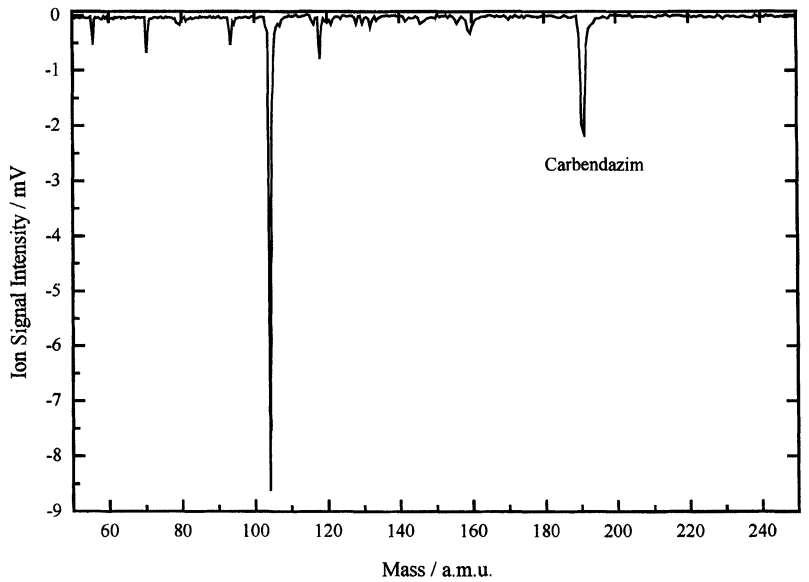

FIGURE 5 Carbendazim TOF mass spectrum obtained from a sample with 49 ng of carbendazim in 152 mg of pepper (0.3 ppm). See text for details.

of the substances contained in the sample. This is of course one of the major advantages of the present technique.

We show here the most interesting results that have allowed us to calculate the detection limits of our system both for the relative carbendazim content in pepper extract and for the absolute quantity of product in a sample. Figure 5 shows the TOF mass spectrum obtained for a sample with 0.3 ppm of carbendazim, (49 ng of carbendazim in 152 mg of pure pepper diluted in 20 mL of acetone). Notice the high level of the carbendazim signal as compared with that of background arising from a pepper extract which contains 152 mg of pure pepper. As mentioned earlier this signal corresponds to a sample whose total amount of carbendazim is 49 ng only. Hence the advantage of using selective ionization for carbendazim analysis is clearly demonstrated. From this signal we can calculate the detection limit of our technique for the analysis of carbendazim. As it is well known, the detection limit of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system, typically this is assumed as three times the noise level [50]. In this spectrum the signal intensity, S, is -2.15×10^{-3} V and the mean noise level, N, was found to be -2.5×10^{-5} V; we will then obtain a S/N = 86 for this sample with 0.3 ppm of carbendazim in pepper. From here we can calculate that our detection limit is better than 0,010 ppm, *i.e.*, 10 ppb of carbendazim in pepper.

This result also shows the great sensitivity of the present method: the sample was deposited on a disc of 7.5 cm diameter (area = 44 cm^2), and the area of desorption was 0.25 cm^2 (ϕ = 0.5 cm); even if one assumes that the laser pulse desorbes completely the carbendazim (which is clearly overestimated), the quantity desorbed will be: 49 ng $\times$ 0,25 cm^2/44 cm^2 = 0.28 ng. The peak signal measured in this spectrum corresponds then to a quantity of carbendazim desorbed per laser pulse below 0.3 ng.

4. CONCLUSIONS

The combination of LD with REMPI-TOF-MS is a suitable method for the determination of carbendazim in agricultural samples. After the optimisation of the experimental conditions and working at the

resonant wavelength carbendazim REMPI spectrum, we have analysed several samples of pepper extract enriched with carbendazim showing excellent results. The detection limit obtained for carbendazim using this technique was of the same order of magnitude as the one published for HPLC, being the amount of sample required to reach this limit far less than in the latter; the technique also shows a great sensitivity as it is able to detect less than 0.3 nanogram of substance.

One clear advantage of the technique is the selective ionisation of minor components in a complex matrix, the great sensitivity and resolution, a major ionisation efficiency and the possibility of simultaneous analysis of all clear components present in a matrix are also significant advantages to be remarked on. Moreover the utilisation of SFE for the sample preparation combined with the use of LD for its desorption allows to facilitate the analysis reducing the time necessary for these operations.

Work is now in progress in our laboratory to perform carbendazim analysis in pepper directly, *i.e.*, without any extraction methods. The results will be the subject of a forthcoming paper.

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