

María Ibáñez · Juan V. Sancho ·
Óscar J. Pozo · Félix Hernández

Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case study

Received: 11 May 2005 / Revised: 6 September 2005 / Accepted: 5 October 2005 / Published online: 1 December 2005
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Abstract Liquid chromatography (LC) coupled to hybrid quadrupole time-of-flight (QTOF) mass spectrometry (MS) is a useful analytical tool in the elucidation and confirmation of transformation products (TPs)/metabolites of pesticides with a wide range of polarity, in both environmental and biological samples. Firstly, the versatility of LC allows the determination of very distinct TPs/metabolites as chromatographic conditions can be easily changed and optimized depending on the analytical problem. Secondly, the mass accuracy provided by the TOF analyser allows the assignment of a highly probable empirical formula for each compound and the differentiation between nominal isobaric compounds. Finally, the possibility of performing MS/MS spectra with accurate mass measurements can be used for the final characterization of the TPs/metabolites detected and for the differentiation of isomeric compounds. In this study, the insecticide diazinon was used as model compound, and its photodegradation and metabolism have been investigated by LC-QTOF-MS. On one hand, environmental spiked water was irradiated with a mercury lamp for 9 days, sampling 3-mL aliquots approximately every 12 h. On the other hand, both *in vitro* and *in vivo* metabolism experiments were carried out with different substrate concentrations and incubation times. After centrifugation, and protein precipitation in the *in vitro* and *in vivo* studies, 50- μ L aliquots of both environmental and biological samples were directly injected into the LC electrospray ionization QTOF system. The most important transformation processes were found to be hydrolysis of the ester moiety, hydroxylation in the aromatic ring or in one of the alkylic groups, oxidation of the sulfur atom on the P=S cleavage or a combination of these processes, with the highest number of compounds being found in the photo-

degradation study. Very polar compounds, such as diethyl phosphate and diethyl thiophosphate, were detected after direct injection of the aqueous sample, which was feasible owing to the characteristics of the LC. In MS mode, mass errors were below 3 mDa, leading to an empirical formula for each compound. MS/MS spectra with accurate mass were used for the final elucidation of the compounds detected.

Keywords Diazinon · Liquid chromatography · Time-of-flight mass analyser · Tandem mass spectrometry · Pesticide transformation products · Pesticide metabolites

Introduction

Currently, there is growing interest in the degradation pathways of organic contaminants such as pesticides, drugs and pharmaceuticals. In the case of pesticides, the determination of transformation products (TPs) in the environment is necessary as some of them could present similar toxicity to or even higher toxicity than the parent compound. Besides, present regulations on drinking water also include TPs within the group of compounds to be monitored. Moreover, the widespread use of pesticides and the potential adverse health effects of pesticide exposure make metabolism studies necessary in order to identify biomarkers of pesticides (normally pesticides metabolites) which might be used for exposure control. The development of analytical methodology for the identification and quantification of pesticide TPs and metabolites is required to properly evaluate the presence and effects of pesticides in both environmental and biological samples.

Data on metabolite formation and on environmental behaviour have to be presented by phytosanitary companies for the official approval and registration of pesticides. For example, study of the environmental fate implies knowledge of the chemical degradation processes in water and soil. Besides, it is also necessary to know the metabolic pathway of the pesticide in animals and plants [1]. However, most of these data have not been published and are difficult to find in the scientific literature.

M. Ibáñez · J. V. Sancho ·
Ó. J. Pozo · F. Hernández (✉)
Research Institute for Pesticides and Water,
University Jaume I,
12071 Castellón, Spain
e-mail: hernandf@exp.uji.es
Tel.: +34-964-728100
Fax: +34-964-728066

One of the commonest approaches to estimate the TP formed in the environment is to study the hydrolytic and/or photolytic degradation of selected pesticides. In relation to metabolism studies, two approaches are the most commonly used: *in vivo* experiments, which allow us to know the metabolic fate in animals, or *in vitro* experiments, e.g. using human liver microsomes. A considerable number of papers can be found in the literature concerning pesticide degradation under environmental, *in vivo* and *in vitro* conditions. However, in most cases these studies focussed on the monitoring of the parent pesticide or a few (normally one or two) preselected metabolites. Conventional chromatographic techniques can be used in these cases to quantify the analytes, such as gas chromatography (GC)-electron capture detection, GC-nitrogen phosphorus detection or liquid chromatography (LC)-UV detection.

However, the elucidation of the metabolites and TPs formed requires the use of more powerful techniques, able to unequivocally confirm the identity of the compound, which in some cases is more difficult owing to the absence of commercial reference standards. The analytical techniques normally applied for elucidation of pesticide metabolites and TPs are ^{13}C -NMR spectroscopy and mass spectrometry (MS; both LC-MS and GC-MS) using a variety of mass analysers. Within the environmental field, some authors have identified the TPs of selected pesticides by using LC-MS/MS [2–4], GC-MS [5–7] or GC-MS/MS [4]. Regarding pesticide metabolism, different techniques have been employed for the identification of the metabolites formed, such as ^{13}C NMR spectroscopy [8, 9] or LC-MS/MS [8].

A useful identification tool in the elucidation of TPs/metabolites is the time-of-flight (TOF) mass spectrometer owing to the increased resolution (more than 5,000 full width at half maximum, FWHM) and high mass accuracy of this analyser (less than 5 ppm), together with its inherent high sensitivity in full-scan mode. LC-TOF-MS has been successfully applied in metabolism studies within the biomedical field for the identification of pharmaceuticals and their metabolites and for the characterization of new drug candidates at the discovery stage [10–13]. However, this technique has scarcely been applied to the elucidation of pesticide TPs in biological and environmental applications mainly owing to its high acquisition cost, as is demonstrated by the limited number of references published. Thus, some authors have used LC-TOF MS for the identification of photodegradation products of alachlor [2, 14], acetochlor [14] and diuron [15]. In these cases, the LC-TOF full-scan data were completed with MS/MS experiments using triple quadrupole [2] or ion trap [15] analysers, and also in-source collision-induced dissociation [2, 14], for structure elucidation of TPs.

Recently, a new powerful identification tool has become available: the hybrid quadrupole TOF (QTOF) mass spectrometer. In addition to the improved characteristics of TOF instruments, they offer the possibility of performing MS/MS acquisitions to obtain product ion spectra with accurate mass, which is sometimes necessary to differentiate between structural isomers. All these characteristics made

QTOF an excellent analyser to study the degradation, transformation and metabolism of organic pollutants at relevant levels. In the environmental field, only a few authors have studied the potential of QTOF for the identification of pesticide TPs. Kouloumbos et al. [3] identified photocatalytic degradation products of diazinon in TiO_2 aqueous suspensions using GC-ion trap MS and LC-QTOF-MS after liquid-liquid extraction with dichloromethane. Recently, our research group has studied the potential of QTOF for the identification of TPs of triazine herbicides after UV exposure [4]. In the biological field, the use of QTOF for the elucidation of pesticides and metabolites is even more limited, and to our knowledge, it has not been reported yet.

The aim of this paper is to show the potential of LC-QTOF MS as a valuable tool for the detection, identification and confirmation of TPs and metabolites of pesticides in laboratory experiments that allow the estimation of degradation under environmental, *in vivo* and *in vitro* conditions. The organophosphorus insecticide diazinon [*O,O*-diethyl *O*-(2-isopropyl-6-methylpyrimidin-4-yl)thiophosphate] has been used as a representative example, although the methodology employed in this work could be applied to many other pesticides, as the majority of TPs and/or metabolites are more properly determined by LC-MS techniques, rather than by GC-MS.

Experimental

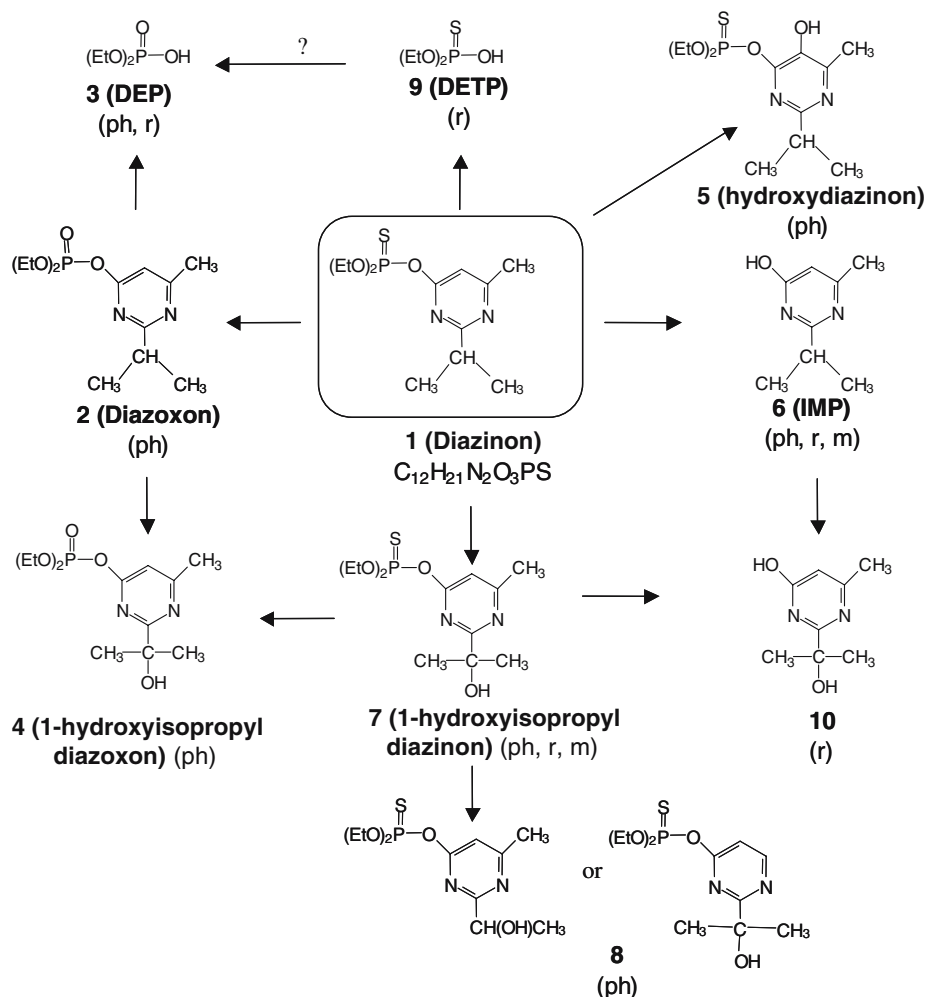
Reagents and chemicals

Diazinon (97.5%) reference standard was purchased from Ehrenstorfer (Augsburg, Germany). The name, chemical structure, elemental composition and exact mass of the protonated molecule for the model compound are shown in Fig. 1. Technical diazinon (60% w/v) used for *in vivo* studies was purchased from Novartis Agro (now Syngenta, Spain). High-performance LC (HPLC) grade methanol, HPLC-grade acetone, analytical-grade formic acid and sodium hydroxide were obtained from Scharlab (Barcelona, Spain). Tetrabutylammonium acetate (TBA, 97%) and sesame oil were obtained from Sigma (St. Louis, MO, USA). HPLC-grade water was obtained by purifying demineralized water in a Nanopure II system (Barnstead, Newton, MA, USA).

Human liver microsomes were obtained from Advancell (Barcelona, Spain). NADP, D-glucose 6-phosphate sodium salt and glucose D-phosphate dehydrogenase were purchased from Sigma. Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were obtained from Scharlab and magnesium chloride from Fluka (Buchs, Switzerland).

Diazinon stock standard solution was prepared dissolving approximately 50 mg of powder, accurately weighted, in 100 mL of acetone to obtain a final concentration of approximately 500 $\mu\text{g}/\text{mL}$. The solutions used for the photodegradation study and for the *in vitro* studies were prepared by diluting this solution with HPLC-grade water.

Fig. 1 Transformation products (TPs)/metabolites identified for diazinon and proposed degradation pathways. *ph* TPs detected in the photodegradation experiment, *r* metabolites detected in *in vivo* experiments with rats and *m* metabolites detected in *in vitro* experiments with microsomes



The solution used for the *in vivo* studies was prepared by dissolving 2 mL of technical product solution (60% w/v) in 8 mL of sesame oil.

Instrumentation

A Waters Alliance 2695 LC system (Waters, Milford, PA, USA) was interfaced to a hybrid QTOF mass spectrometer (QTOFI, Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface. The LC separation was performed using an X-Terra C_{18} 5 μ m, 2.1 mm \times 250 mm (Waters) at a flow rate of 200 μ L/min. The mobile phase used was an aqueous 0.01% HCOOH/methanol-0.01% HCOOH gradient where the percentage of organic solvent was changed linearly as follows: 0 min, 5%; 20 min, 95%; 23 min, 95%; 24 min, 5%; 29 min, 5%. The separations were completed within 26 min.

The drying gas as well as the nebulizing gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, UK). The nebulizer gas flow was set to approximately 15 L/h and the desolvation gas flow to 500–600 L/h. TOF-MS resolution was approximately 5,000 (FWHM). MS data were ac-

quired over the *m/z* range 60–600. The multichannel plate detector potential was set to 2,700 and to 2,800 V in positive and negative electrospray ionization (ESI) modes, respectively.

For operation in MS/MS mode, the collision gas was argon (99.995%, Carburos Metalicos, Valencia, Spain) with a pressure of approximately 2.5×10^{-5} mbar in the collision cell. A cone voltage of 25 V and a capillary voltage of 3.5 kV were used in both positive and negative ionization modes. The interface temperature was set to 350°C and the source temperature to 120°C. Dwell times of 1 s/scan were chosen. A solvent delay of 8 min was selected to perform a cleanup using the built-in divert valve monitored by time-controlled contact closures using Masslynx NT version 4.0 software. A suitable MS profile was used.

Calibration experiments were performed daily using a model 11 single-syringe pump (Harvard, Holliston, MA, USA), directly connected to the interface. Calibration in both positive and negative ESI modes from 60 to 600 *m/z* was conducted with a mixture of 0.1 M NaOH-10% HCOOH (50:50) diluted (1:25) with acetonitrile-water (80:20) at a flow rate of 10 μ L/min.

A 1 μ g/mL solution of 3,5-diiodo-L-tyrosine in methanol, used as lock mass, was introduced after the column

using a model 2150 isocratic HPLC pump from LKB (Bromma, Sweden) at a flow rate of 100 $\mu\text{L}/\text{min}$ by means of a PEEK T-union [4].

Degradation/metabolism studies

Photodegradation

The irradiation procedure applied in this work has been previously used in our laboratory [12]. Briefly, irradiation was performed using a mercury vapour lamp with its main output at 254 nm. The 500-mL surface water samples were kept in glass beakers. The distance from the lamp to the surface of the samples was approximately 15 cm. Irradiation was carried out in a fume hood at room temperature over a period of approximately 193 h under constant stirring of the samples. Prior to the photolysis experiment, a 500-mL surface water sample was spiked at 500 ng/mL with diazinon, by adding 1 mL of 500 $\mu\text{g}/\text{mL}$ standard solution. Additionally, a 500-mL non-spiked water sample was irradiated under the same conditions and used as a control sample for all experiences. Before irradiation ($t=0$), a 3-mL water aliquot was analysed, and during irradiation 3-mL water samples were taken from the beakers at different time intervals, centrifuged for 10 min (Angular 6 centrifuge, Selecta, Barcelona, Spain) to remove possible solid particulate matter, and analysed by direct injection of 50 μL of the supernatant into the LC-QTOF mass spectrometer. Protective glasses for personal protection were used in order to avoid UV exposure.

Human liver microsome incubation (in vitro studies)

Firstly, the NADPH-generating system was prepared, consisting of 1 M MgCl_2 , 100 mM phosphate buffer (pH 7.4), 100 mM NADP, 500 mM glucose 6-phosphate and 1.5 IU glucose D-phosphate dehydrogenase (final volume 0.5 mL). After 10-min prewarming at 37°C in presence of microsomes, phosphate buffer (100 mM, pH 7.4) and the substrate at a final concentration of 1 $\mu\text{g}/\text{mL}$, the incubation was initiated by addition of the NADPH-generating system. After 15, 30, 45, 60, 90, 120 and 150 min the reaction was stopped by addition of 0.5 mL of acetonitrile. Finally, samples were centrifuged and 50 μL of the supernatant was directly injected into the LC-QTOF mass spectrometer.

Additionally, and for each time, a control sample (without microsomes) and a blank sample (without substrate) were prepared in order to differentiate between metabolites originating from the microsomes and possible metabolites or TPs originating from the incubation procedure.

In vivo studies

Male Sprague-Dawley rats, weighting 300–350 g, were purchased from Janvier Spain (Barcelona, Spain). Upon

receipt, the animals were examined and found to be in good health. Before use, the animals were acclimated to the laboratory environment for 1 week. The rats were housed individually in polypropylene cages at 22 \pm 2 °C with 12-h light-and-dark cycles. Standard laboratory rodent chow and tap water were provided ad libitum throughout the study.

Diazinon, as a solution in sesame oil, was administered to rats via intraperitoneal administration, in target doses of 1,250 and 125 mg/kg of body weight (dose volume of 10 mL/kg). The control animals were given an equal volume of vehicle by the same method.

At the specified time after dose administration (3, 9 and 24 h), the rats were killed by decapitation under anaesthesia. Trunk blood (1.5 mL) was collected in vials containing heparin. Samples were centrifuged at 7,500 rpm (5,031 g) for 5 min and plasma was frozen at -80 °C. All experimental procedures complied with the European Community Council Directive (86/609/ECC) for the use of laboratory animal subjects.

Before analysis, protein precipitation was carried out with acetonitrile. After centrifugation in a high-frequency induction motor centrifuge (Centronic B.L., Selecta), 50 μL of the supernatant was directly injected in the LC-QTOF mass spectrometer.

Data processing

For the determination of accurate mass, the experiments were acquired in continuum in both positive and negative ionization modes and afterwards processed by the application manager MetaboLynx version 4.0 (Micromass). MetaboLynx compares two LC-MS data files (one corresponding to the analyte sample and the other corresponding to the blank sample) and highlights differences due to the presence of new compounds, which could be attributed to transformation processes. For all compounds detected by MetaboLynx, the exact mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Where abundant peaks were observed (more than 400 counts/s), the digital dead time correction algorithm could not correct the deviations obtained, and therefore, data were taken from the peak tail, i.e. at lower signal intensity.

On the basis of the accurate mass, possible elemental compositions of the peaks of interest were calculated using the elemental composition calculator, within the MassLynx software, with a maximum deviation of 5 mDa from the measured exact mass. The parameter settings were C 0–15, H 0–50, N 0–5, O 0–8, S 0–1 and P 0–1. The applied double-bond equivalent filter was set between -0.5 and 10. The maximum and minimum parameters were restricted as a function of the structure of diazinon.

The possibility of performing MS/MS experiments helped us to elucidate the structure of several TPs as we obtained a product ion spectrum with the exact mass of fragments. In this case, the exact mass of the precursor ion was used as a lock mass.

Results and discussion

Metabolites and photodegradation products identified

The most important processes found to occur in the degradation/metabolism of diazinon were *hydrolysis* of the ester moiety, *hydroxylation* and *oxidation*, as can be seen in Fig. 1. A combination of these processes was also observed.

The hydrolysis of the ester moiety gave 2-isopropyl-6-methyl-4-pyrimidin-4-ol (IMP, compound **6**) and diethyl thiophosphate (DETP, compound **9**). In the *in vitro* study, although IMP was detected, no DETP was found, possibly owing to the low sensitivity for alkylphosphates in electrospray interfaces. However both, IMP and DETP were detected in the *in vivo* study. Moreover, diethyl phosphate (DEP, compound **3**) was also found, which could result from the hydrolysis of diazoxon (compound **2**) or from the oxidation of DETP. In the photodegradation study, only IMP and DEP were detected, possibly owing to the oxidative conditions of these experiments, which favoured the oxidation previous to the hydrolysis.

As can be deduced from Fig. 1, hydroxylation can occur in the aromatic ring or in one of the alkyl groups. In both *in vivo* and *in vitro* studies, the hydroxylation was only observed in the isopropyl group (1-hydroxy isopropyl diazinon, compound **7**) meanwhile hydroxylation occurred in both the isopropyl group and the aromatic ring (compounds **5**, **7** and **8**) in the photodegradation study.

Finally, the oxidation of the sulfur atom on the P=S cleavage was observed. Although the oxidation products diazoxon and 1-hydroxy isopropyl diazoxon (compound **4**) were only identified in the photodegradation study, the occurrence of DEP in the *in vivo* studies indicated that this metabolic pathway may also occur in rats.

A combination of several processes was observed in some cases, such as hydroxylation plus oxidation (1-hydroxy isopropyl diazoxon) or hydroxylation plus hydrolysis (compound **10**).

Demethylation was only observed in the photodegradation study for 1-hydroxy isopropyl diazinon to give compound **8**.

These results are consistent with others previously reported for environmental [3, 16–18] and biological [19–21] samples. However, to our knowledge, compounds **5** and **10** have not been previously reported in the literature.

Photodegradation curves for diazinon and some of its TPs are shown in Fig. 2, where separate signal normalization of each product to its maximum value was used and is represented as a function of irradiation time. This approach was used owing to the unavailability of standards for all TPs detected as well as because of the low linear dynamic range of QTOF instruments, which made difficult the correct quantitation even when reference standards were available. As depicted in Fig. 2, simultaneously to the disappearance of diazinon the formation of several peaks is observed, giving oxidated and hydroxylated products

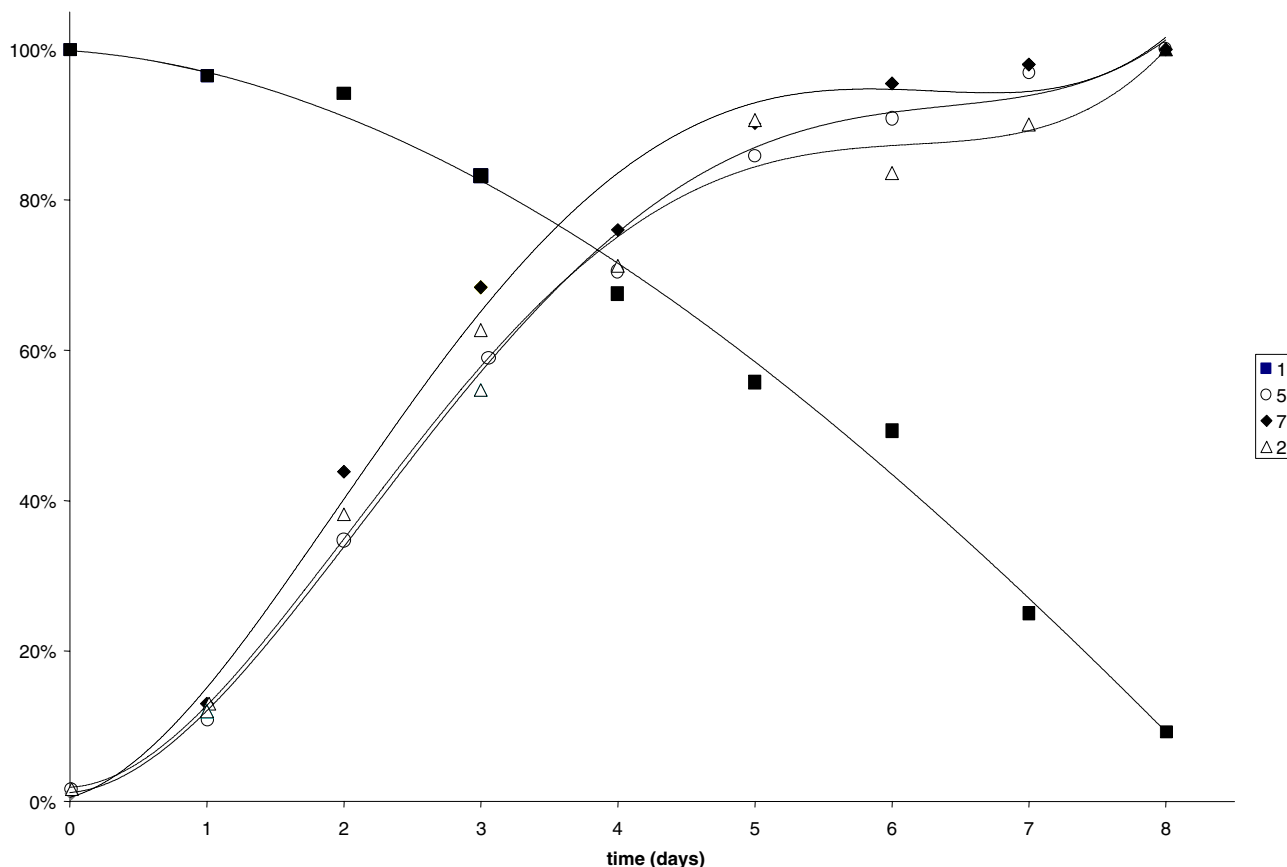


Fig. 2 Degradation curves for diazinon (**1**) and some of its more relevant transformation products (**5**, **7**, **2**)

mainly. Compounds **2**, **5** and **7** were the main phototransformation products found. Under the experimental conditions of this work, parent diazinon was degraded in 1 week down to about 40%.

LC considerations

Although GC has been used to determine pesticides and their TPs, LC is found to be more adequate as the majority of the TPs are more polar than their parent pesticides, less volatile and often also thermolabile. Besides, the feasibility of injecting directly an aqueous sample (such as water or plasma) into the LC column minimizes the risk of losses for TPs that could occur during the sample pretreatment, providing that sensitivity is sufficient for the objectives pursued. Otherwise, a preconcentration step would be required. Moreover, chromatographic conditions can be easily changed to obtain better chromatography when required.

These advantages were observed in this work, where the inadequate chromatographic compounds detected by MetaboLynx at m/z 169.0 and 153.0 in the negative ionization mode (Fig. 3a) prevented us from confirming that they were diazinon metabolites. Although the accurate masses obtained were in accordance with the exact masses of DEP and DETP, these types of broad peaks might also have been produced by the effect of the mobile-phase gradient. These two alkylphosphates and other similar compounds were previously studied in our laboratory [22, 23], and we

proved the need to add an ion-pairing reagent into the mobile phase and/or the sample vial in order to obtain adequate chromatographic behaviour. Thus, after adding TBA into the vial as the ion-pairing reagent, satisfactory chromatography was obtained (Fig. 3b), confirming the presence of the metabolite DEP, although a decrease in sensitivity was observed. This example shows the potential of LC for the determination of polar metabolites, as the experimental conditions can be easily and efficiently modified depending on the analytical problem to be solved. Additionally, the possibility of injecting directly aqueous samples can be pointed out, which allowed us to determine metabolites as polar as alkylphosphates, which would have been lost by conventional sample pretreatment procedures such as solid-phase extraction or liquid-liquid extraction. In fact, these compounds were not reported by Kouloumbos et al. [3] when studying the degradation of diazinon by LC-MS, possibly owing to the loss of these polar compounds after extraction with dichloromethane. But even if these metabolites were recovered during the sample treatment, their determination by GC-based methods would require a derivatization step, which would increase analysis time.

TOF-MS considerations

Undoubtedly, MS is one of the best techniques for the elucidation of metabolites owing to its sensitivity, universality and the sample information provided. Although

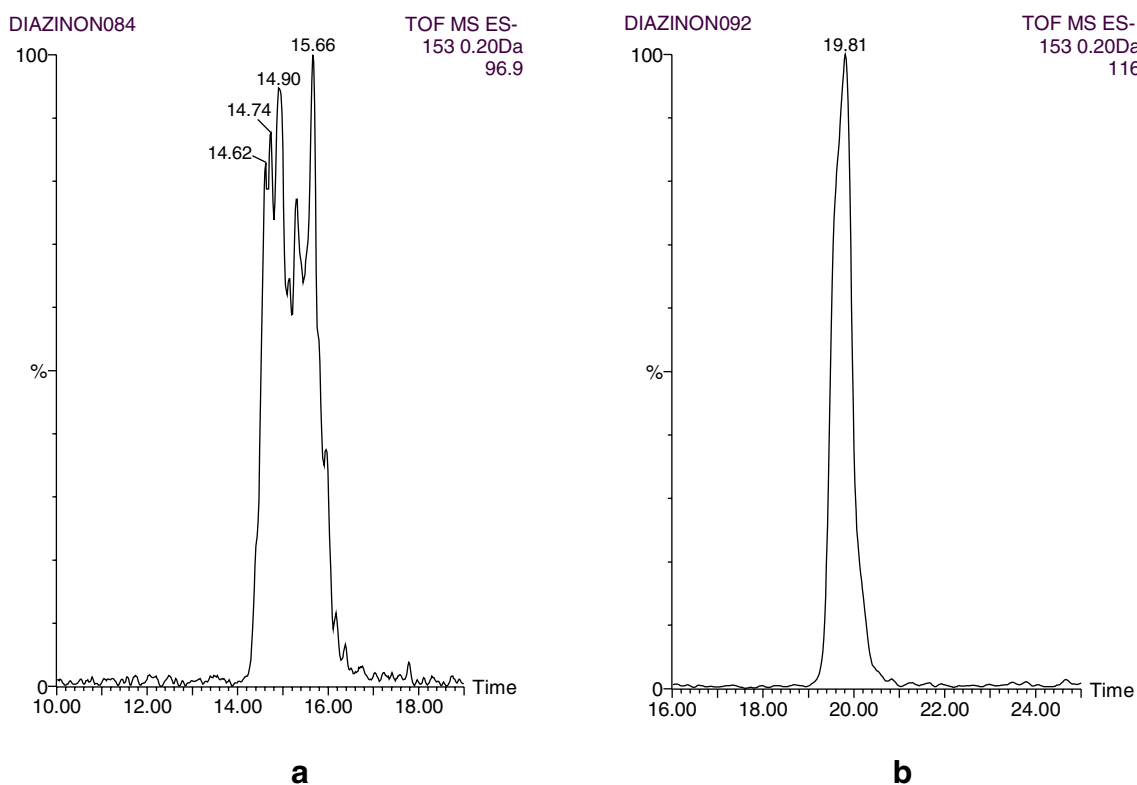


Fig. 3 Liquid chromatography (LC)-mass spectrometry (MS) chromatograms (mass window 0.2 Da) for diethyl phosphate **a** without tetrabutylammonium acetate (TBA) in the sample vial and **b** with 4 mM TBA in the sample vial

quadrupole instruments can be a useful tool for the determination of the elemental composition, these instruments present an important drawback when dealing with isobaric compounds. The low resolution of these analysers avoids the differentiation of compounds with m/z differing by less than 1 Da. Although this limitation could be minimized with low-resolution MS/MS instruments, the use of TOF analysers allows an easier and more confident estimation of their elemental composition owing to the high resolution and mass accuracy power. Moreover, the sensitivity achieved by TOF instruments in scan mode is superior than that of quadrupoles, facilitating the detection of less abundant metabolites.

In this work, the potential of TOF-MS was proved in the differentiation of compounds **1** and **4**. Both compounds have the same nominal mass (m/z 305) and, therefore, they were indistinguishable by quadrupole instruments. When studying the exact mass of both compounds (m/z 305.1266 for compound **4** and m/z 305.1089 for compound **1**), we obtained a difference of 17.7 mDa, corresponding to the elemental composition $C_{12}H_{22}N_2O_5P$, for compound **4**, and $C_{12}H_{22}N_2O_3PS$, for compound **1**. This mass difference was found to be sufficient, as when applying the elemental composition calculator with the selected parameters (maximum deviation 5 mDa, see “Experimental” section) only one hit appeared, allowing us to assign the elemental composition to each of these compounds.

The results obtained in TOF experiments are summarized in Table 1. This table shows the parent compound and the nine TPs detected. When accurate mass was calculated by averaging the mass of all the products detected, deviations lower than 2 mDa were obtained for every compound except for compounds **1**, **7** and **10**, but still with deviations lower than 3 mDa. With these results, one can be confident about the elemental composition given for each analyte.

QTOF-MS considerations

Despite the capability of TOF analysers to distinguish isobaric compounds (mass differences lower than 1 Da), their usefulness is limited when dealing with isomers, as they present the same molecular composition and, consequently, the same exact mass. However, hybrid QTOF instruments give the possibility of performing MS/MS acquisitions to obtain product ion spectra with accurate mass, which can help to fully differentiate between isomeric analytes in a more confident way than when using low-resolution MS/MS instruments.

Thus, although the calculated composition for compound **4** ($C_{12}H_{22}N_2O_5P$) suggested this compound to be a monohydroxylated product of diazoxon, with these data it is not feasible to know whether the TP corresponded to (1) hydroxylation of the aromatic methyl group (site 1 in Fig. 4), (2) hydroxylation of the ethyl group of the thiophosphoric part (site 2 in Fig. 4), (3) hydroxylation of the primary or secondary carbon atoms of the isopropyl group (sites 3 and 4 in Fig. 4, respectively) or (4) hydroxylation of the aromatic group (site 5 in Fig. 4). In order to elucidate this metabolite, MS/MS experiments were carried out.

The product ion spectra of compound **4** (Fig. 4b) showed a fragment at m/z 287.1186 ($\Delta mDa=2.5$), corresponding to the loss of water from the precursor ion m/z 305.1266 (Table 2. Note that the mass fragments shown in this table are theoretical masses.), showing that the hydroxylation could not occur either in the aromatic or in the methyl group, owing to the absence of α hydrogens (sites 1 and 5 in Fig. 4). This fact was corroborated in the MS/MS spectra of compound **5** (Table 2), where no loss of water was observed. The abundant fragment at m/z 169 might be obtained after the loss of the intact diethyl hydrogen phosphonate part (169.0992, $C_8H_{13}N_2O_2$), suggesting that oxidation took place on the isopropyl group of the pyridine

Table 1 Transformation products/metabolites identified for diazinon. Exact mass measurements and mass errors for the proposed compounds

Proposed compound	Retention time (min)	Ionization modes	Elemental composition $[M+H]^+/[M-H]^-$	Theoretical mass $[M+H]^+/[M-H]^-$	Deviation (mDa)		
					Photodegradation ^a	In vivo ^a	In vitro ^b
1 (parent)	25.3	ESI+	$C_{12}H_{22}N_2O_3PS$	305.1089	-0.8	2.6	1.0
2	22.3	ESI+	$C_{12}H_{22}N_2O_4P$	289.1317	-0.9	-	-
3	11.9	ESI-	$C_4H_{10}O_4P$	153.0317	-0.3	-1.1	-
4	20.3	ESI+	$C_{12}H_{22}N_2O_5P$	305.1266	0.1	-	-
5	23.1	ESI+	$C_{12}H_{22}N_2O_4PS$	321.1038	0.3	-	-
	23.1	ESI-	$C_{12}H_{20}N_2O_4PS$	319.0882	-1.0	-	-
6	13.7	ESI+	$C_8H_{13}N_2O$	153.1028	-0.2	-2.0	1.5
		ESI-	$C_8H_{11}N_2O$	151.0872	-	-1.0	-
7	23.7	ESI+	$C_{12}H_{22}N_2O_4PS$	321.1038	2.7	0.4	-0.5
8	22.3	ESI+	$C_{11}H_{20}N_2O_4PS$	307.0881	-0.4	-	-
9	24.5	ESI-	$C_4H_{10}O_4PS$	169.0089	-	-1.0	-
10	12.9	ESI+	$C_8H_{13}N_2O_2$	169.0977	-	2.9	-
		ESI-	$C_8H_{11}N_2O_2$	167.0821	-	-0.6	-

^aData obtained after 24 h of exposure

^bData obtained after 2.5 h of incubation

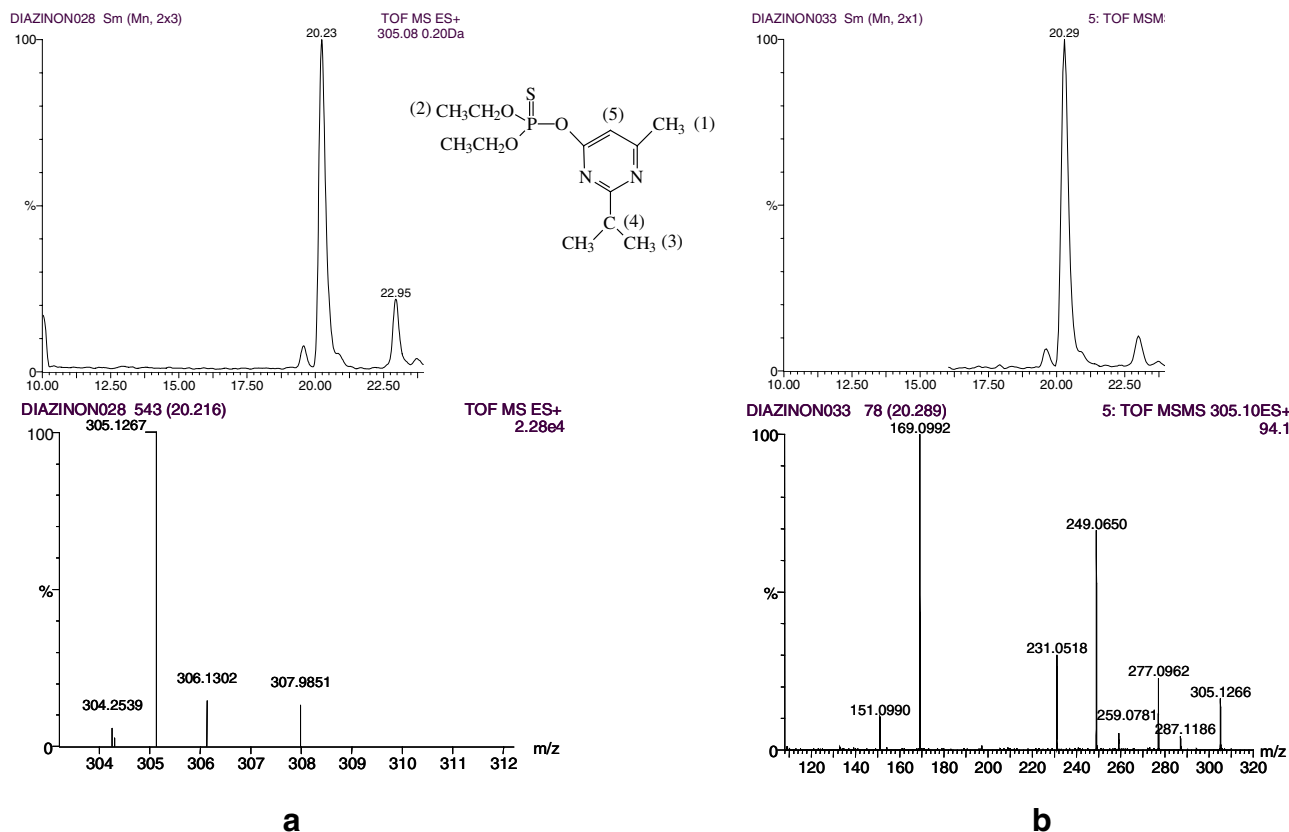


Fig. 4 Elucidation of compound **4** (retention time 20.2–20.32 min): **a** time-of-flight (TOF) MS chromatogram (top) and positive ion electrospray full-scan mass spectrum (bottom); **b** LC-TOF MS/MS chromatogram (top) and positive ion electrospray product ion spectra (bottom). 1–5 possible hydroxylation sites

moiety of the molecule (sites 3 and 4 in Fig. 4), or after the loss of the alkylated aromatic ring (169.0266, $C_4H_{10}O_5P$), suggesting the possibility of hydroxylation in the phosphoric part (site 2 in Fig. 4). Using a low-resolution MS/MS instrument it would not be feasible to distinguish where the hydroxylation took place. However, with the QTOF we observed a fragment at m/z 169.0977, that differed by only 1.5 mDa from $C_8H_{13}N_2O_2$ (m/z 169.0992) and 76.6 mDa from $C_4H_{10}O_5P$ (169.0266), showing that the hydroxylation occurred in the isopropyl group. Nevertheless, in spite of these data we could not distinguish whether the hydroxylation had taken place in the primary or in the secondary carbon atoms of the isopropyl group (sites 3 or 4 in Fig. 4, respectively). Kouloumbos et al. [3] observed an ion of m/z 275 corresponding to the loss of a formaldehyde molecule from the protonated molecule in the MS/MS spectra of the 2-hydroxyisopropyl diazoxon. However, in our work this fragment was not observed, suggesting that hydroxylation occurred on the secondary carbon atom of the isopropyl group of diazinon. Compound **4** was identified as 1-hydroxyisopropyl diazoxon. The other fragments observed were m/z 277.0962 ($\Delta mDa=0.9$), corresponding to the loss of an ethylene group, m/z 249.0650 ($\Delta mDa=1.0$), corresponding to the loss of two ethylene groups, and m/z 259.0781 ($\Delta mDa=6.7$), 231.0518 ($\Delta mDa=1.7$) and 151.0990 ($\Delta mDa=1.2$), corresponding to losses of water from fragments at m/z

277.0962, 249.0650 and 169.0992, respectively. The identity of this compound could not be further validated, owing to the unavailability of a reference standard. In a similar way, all TPs of diazinon obtained under the different experimental conditions assayed were identified (Fig. 1).

As can be seen in Table 1, both positive and negative ionization modes were used in this work. Acquisition in negative mode was required for detection of alkylphosphates (compounds **3** and **9**), while for other compounds (compounds **5**, **6** and **10**) acquisition in both modes gave complementary information for a better assignment of the structure.

In spite of the evident advantages of QTOF-MS shown in this paper, there is a limitation that should be taken into account, and it is derived from the limited understanding of the fragmentation rules in MS/MS of (de)protonated molecules. In some cases, especially when dealing with poor MS/MS spectra, the unequivocal elucidation of the structure of a compound is not possible. For example, we could not distinguish where the demethylation process had occurred in compound **8** (Fig. 1). In these cases, if standards are commercially available, both the retention time and MS/MS data can be used as a confirmatory tool. However, when reference standards are not available and their synthesis is not feasible, the combination of several techniques may help in this difficult task. For example, since the commercial introduction of LC-NMR systems, this hyphenated

Table 2 Transformation products/metabolites identified in the degradation studies of diazinon. Mass fragments and mass errors for the proposed compounds obtained by liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry

Compound	Ionization mode	Precursor ion	Theoretical mass fragments	Deviation (mDa)	
				Photodegradation ^a	In vivo ^a
1 (parent)	ESI+	305.1089	169.0799	1.9	-1.3
			153.1028	1.9	-0.8
			124.9826	2.4	0.1
			114.9619	0.4	0.3
2	ESI+	289.1317	261.1004	0.1	
			233.0691	-2.6	
			153.1028	1.2	
			135.0922	0.8	
3	ESI-	153.0317	125.0004	-0.4	-7.6
			78.9585	0.8	-1.1
4	ESI+	305.1266	287.1161	2.5	
			277.0953	0.9	
			259.0848	-6.7	
			249.0640	1.0	
			231.0535	-1.7	
			169.0977	1.5	
			151.0871	1.2	
5	ESI+	321.1038	293.0725	-1.2	
			185.0749	2.0	
			169.0977	2.2	
	ESI-	319.0882	291.0568	2.8	
			229.0412	-4.0	
			183.0592	-1.1	
6	ESI+	153.1028	137.0715	1.6	0.3
			84.0575	-5.2	0.8
	ESI-	151.0872	135.0558		-0.7
			123.0558		1.8
			109.0402		0.6
			93.0092		0.3
			82.0293		0.2
7	ESI+	321.1038	303.0932	0.5	-2.3
			275.0619	-1.0	-3.5
			247.0306	-1.8	-5.1
			185.0749	-2.3	-9.3
			169.0977	0.7	-0.5
			167.0643	-0.7	-6.5
			151.0871	0.5	-6.5
8	ESI+	307.0881	289.0776	0.9	
			261.0463	0.4	
			233.0150	-2.9	
			171.0592	1.6	
			153.0486	0.6	
			137.0715	1.0	
9	ESI-	169.0089	140.9775		0.5
			94.9357		-0.5
10	ESI+	169.0977	151.0871		0.7
			84.0449		6.1
	ESI-	167.0821	109.0402		-0.2

^aData obtained after 24 h of exposure

technique has been mainly applied to the structural elucidation of metabolites in drug development studies and to the identification of natural compounds in plant extracts,

giving optimum structural information when both LC-MS and LC-NMR are used together. However, LC-NMR presents poor sensitivity, generally 3 orders of magnitude

worse than LC-MS, which limits its applications in some cases. Another complementary tool that might be useful in the elucidation of the structure of potential TPs/metabolites is GC-electron impact (EI)-MS. In this case, the use of an EI source would allow the measurement of non-ESI ionizable compounds followed by an easier identification using spectra matching in commercial libraries, provided that TP/metabolite spectra are available.

Conclusion

This work demonstrates that LC coupled to hybrid QTOF-MS is a powerful analytical tool for the elucidation of metabolites and TPs of selected pesticides. The versatility of LC, together with the high mass accuracy, good sensitivity and possibility of performing MS/MS experiments with a QTOF instrument allows the detection and identification of TPs/metabolites after direct injection of aqueous sample extracts, without the need of laborious sample pretreatment, minimizing possible losses of analytes in the analytical procedure. However, this approach presents some limitations, as the interface selected restricts the application to those compounds ionizable with this interface, normally from medium polarity to ionic. Moreover, the achievement of information-rich product ion spectra becomes a relevant factor in the elucidation process on some occasions.

The main TPs identified in the photodegradation study of diazinon were IMP and two TPs produced by hydroxylation of the isopropyl group of both diazinon (1-hydroxyisopropyl diazinon) and its oxygen analogue diazoxon (1-hydroxyisopropyl diazoxon). As experiments were carried out under oxidative conditions, it would be interesting to extend the photodegradation experiments using a different source (e.g. xenon lamps) in order to better simulate sunlight conditions and to obtain more realistic results. Regarding *in vitro* studies, despite the use of different substrate concentrations and incubation times, only two metabolites, corresponding to hydrolysis (IMP) and hydroxylation (1-hydroxyisopropyl diazinon) processes were observed. Metabolism studies were completed by performing *in vivo* experiments, which led to the formation of metabolites corresponding to hydrolysis and hydroxylation, as well as oxidation of diazinon.

Because diazinon metabolites/TPs are more polar than the parent compound, they must be more water-soluble and possibly more mobile and might have greater potential for leaching in the soil-water environment. Therefore, they might probably be found in surface and ground water at higher concentrations than the parent compound. It would be interesting to investigate within the water monitoring programmes the most relevant TPs found in the photodegradation study: diazoxon (compound 2), 1-hydroxyisopropyl diazinon (compound 7) and hydroxydiazinon (compound 5). In the case of human biofluids, IMP

(compound 6) and 1-hydroxyisopropyl diazinon (compound 7) seem to be adequate biomarkers for evaluation of human exposure to this pesticide.

Acknowledgements The authors are very grateful to Serveis Centrals d'Instrumentació Científica (SCIC) for use of the Q-TOF hybrid QTOF mass spectrometer and to Marta Miquel and Carlos Aragonés from the Physicobiology Department, University Jaume I, for their excellent technical assistance in the *in vivo* studies. This work forms part of the research project "Aplicación del acoplamiento cromatografía líquida-espectrometría de masas (LC-MS/MS y LC-QTOF MS) a la identificación de metabolitos de plaguicidas en muestras de interés ambiental y toxicológico" (Ref: BQU2003-02685) developed with financial support of the Ministerio de Ciencia y Tecnología, Spain. M.I. is very grateful to Generalitat Valenciana for her predoctoral grant.

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