

# Monitoring of 6-chloronicotinic acid in human urine by gas chromatography-tandem mass spectrometry as indicator of exposure to the pesticide imidacloprid

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A new analytical method for determining 6-chloronicotinic acid (6-CINA) in human urine is proposed. 6-CINA is the main metabolite in warm-blooded animals after exposure to the insecticide imidacloprid. 6-CINA was extracted from human urine using solid phase extraction (SPE) with laboratory-made cartridges of Amberlite XAD-4. A clean-up step and a derivatization process were carried out prior to gas chromatography-tandem mass spectrometric (GC-MS-MS) determination. A study on the influence of pH in the extraction process revealed that it affects the analyte extraction efficiency. A working pH zone was defined between 0.8 and 2.8. Calibration curves were studied in the concentration range of 0.5–100 ng mL<sup>-1</sup> and showed good linearity. Limits of detection and determination of the method were 16 and 56 pg mL<sup>-1</sup> respectively. The mean recovery at 10 and 100 ng mL<sup>-1</sup> was between 97.2 and 102.1% and the repeatability was lower than 5.4% in all cases. The analysis of urine samples of five agricultural workers from Almería (Spain) did not detect the metabolite.

## Introduction

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine] is a chloronicotinyl insecticide, one of a new group of active ingredients with novel modes of action.<sup>1</sup> Owing to its insecticidal effectiveness and its safety for humans and the environment, it is extensively used in agricultural areas like Almería (Spain).

Physiologically, imidacloprid interferes with neural functions as do organophosphate, carbamate, and pyrethroid insecticides. Different from the later pesticides, it acts as an agonist of acetylcholine by binding to nicotinic acetylcholine receptors on the postsynaptic membrane.<sup>2–4</sup> In mammals, after an oral administration of imidacloprid, 70–80% of the total amount is eliminated very quickly *via* the urine and faeces. Imidacloprid is nevertheless very thoroughly metabolized. The most important metabolic steps are hydroxylation at the imidazolidine ring to 1-(6-chloro-3-pyridylmethyl)-2-(hydroxymino)-3,4-didehydroimidazolidene (6-CIPHD), hydrolysis to 6-chloronicotinic acid (6-CINA), loss of the nitro group with formation of the guanidine and conjugation of the 6-CINA with glycine.<sup>5</sup> Fig. 1 shows the structure of imidacloprid and the major initial metabolic pathways.

Several methods have been published for the determination of imidacloprid residues in different matrices, such as waters,<sup>6–8</sup> vegetables,<sup>9–11</sup> soils,<sup>12–16</sup> and greenhouse air<sup>17</sup> but no methods have been published for the determination of residues of this insecticide or its metabolites in biological samples.

The determination of trace amounts of a pesticide and/or metabolite in biological matrices such as urine presents difficulties that must be solved,<sup>18</sup> for example, the presence of large amounts of substances that can interfere with the analytical signal of the target analyte. Such interferences must be minimized using selective analytical techniques. Recently, the use of ion trap mass spectrometry coupled to GC has shown great promise for the determination of very low levels of pesticides and metabolites in biological samples using tandem mass spectrometry (MS-MS).<sup>19–22</sup> This technique offers higher sensitivity than full scan mass spectrometry and more qual-

itative information than selected ion monitoring mass spectrometers.

This paper proposes a new analytical method for determining 6-CINA in human urine using SPE and GC-MS-MS. The method has been validated with fortified urine samples and real samples from volunteers that work in greenhouses in Almería (Spain).

## Experimental

### Chemicals

Pesticide grade hexane, dichloromethane, diethyl ether, acetone and methanol were obtained from Panreac (Barcelona, Spain).

Organic free water was prepared by distillation and then by passing through Milli-Q SP Columns (Millipore, USA).

Analytical grade sulfuric acid (98% purity), anhydrous sodium sulfate and potassium carbonate (for analysis of residues) were purchased from Panreac.

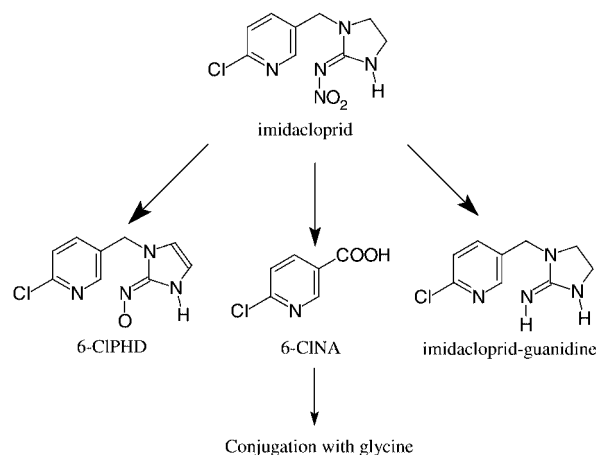


Fig. 1 Structure of imidacloprid and its main metabolic pathways in mammals.

1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) and diisopropylcarbodiimide (DIC) were obtained from Aldrich (Steinheim, Germany). Sigmasil A was purchased from Sigma (St. Louis, MO, USA). They were stored in a refrigerator (4 °C) and used as received.

Pure 6-chloronicotinic acid and nicotinic acid (internal standard, IS) were obtained from Riedel-de-Haën (Seelze, Germany). Individual stock solutions of the metabolite and IS at 300 µg mL<sup>-1</sup> were prepared in acetone and stored in a freezer at -30 °C. The working solutions were obtained by appropriate dilution of the stock solution with acetone and stored in a refrigerator (4 °C). The IS was diluted to 3 µg mL<sup>-1</sup> with acetone.

Amberlite XAD-4 for SPE was obtained from Supelco (Bellefonte, PA, USA). XAD-4 columns were prepared by packing Pasteur pipettes (diameter, 0.5 mm; length, 10 cm) with 700 mg of the resin previously cleaned using diethyl ether for 16 h in a Soxhlet extractor operating at 20 min per cycle. The packed pipettes were dried under a nitrogen current and stored in the dark.

## Apparatus

A Saturn 2000 gas chromatograph-ion trap mass spectrometer (Varian Instruments, Sunnyvale, CA, USA) was used. The gas chromatograph was fitted with an 8200 autosampler, a split/splitless temperature programmable injector 1078 and operated in splitless mode. The column used was a DB5-MS (30 m × 0.25 mm id) with a 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). The ion trap mass spectrometer was operated in the electron ionization mode (EI, 70 eV) and the MS-MS option was used. The data handling system had an EI-MS-MS library especially created for storing the MS-MS target analyte spectra under our experimental conditions. An additional commercial EI-MS library was available.<sup>23</sup> The carrier gas used was helium (purity N50).

A test tube shaker with a variable speed controller was purchased from Ika-Works (Wilmington, NC, USA).

## Sample collection

Urine samples from five volunteer agricultural workers were collected and stored in sterilized containers. Samples were frozen immediately and kept at -30 °C until analyzed.

## Urine extraction procedure

A 3 mL aliquot of urine was placed in a screw capped glass centrifuge tube to which was added 100 µL IS and 50 µL H<sub>2</sub>SO<sub>4</sub> (5 M). The tube was heated at 90 °C in a water bath for 1 h. After cooling to room temperature, the sample was passed through the Amberlite XAD-4 cartridge previously washed with 5 mL of diethyl ether, dried and conditioned with 7 mL of methanol and 4 mL of water (pH = 1.3) in that order. (Note: the solid phase was not allowed to dry during conditioning). To carry out a clean-up step, 2 mL of water (pH = 1.3) and 2 mL of hexane were added in that order. Analyte was eluted from the solid phase with 7 mL diethyl ether which were passed through anhydrous Na<sub>2</sub>SO<sub>4</sub> to remove water. It was contained in a clean Pasteur pipette capped in the bottom with a small amount of glass wool. The solvent was evaporated under a stream of nitrogen without heat and the residue was re-dissolved in 1 mL of hexane.

For derivatization, 10 µL of HFIP were added to the extract with gentle mixing followed by addition of 15 µL of DIC. After shaking for 3 min with the test tube shaker at 1100 rpm, the extract was washed with 1 mL of 5% aqueous potassium carbonate solution to neutralize the excess derivatizing agent.

The organic layer was transferred to a 2 mL autosampler vial for analysis.

## GC-MS-MS conditions

A 1 µL aliquot of the extract was injected into the gas chromatograph with the split closed for 1.5 min. The carrier gas flow was set at 36.6 cm s<sup>-1</sup> (150 °C oven temperature) and the injector temperature was programmed from 90 °C (held 0.1 min at 90 °C) to 280 °C at 200 °C min<sup>-1</sup> (held for 15 min at 280 °C). The oven temperature was modified from 60 °C (held for 1.5 min at 60 °C) to 270 °C at 20 °C min<sup>-1</sup> (held for 5 min at 270 °C).

The mass spectrometer was calibrated weekly. The ionization mode was electron ionization (EI) with an electron energy of 70 eV, the multiplier voltage was set at 1700 V, A/M amplitude voltage was 4.0 V, temperatures were fixed for trap, manifold and transfer-line at 200, 45 and 280 °C, respectively. The emission current was set at 80 µA, the automatic gain control (AGC) was switched on and the AGC target was fixed at 5000 counts. The MS-MS conditions are summarized in Table 1.

## Results and discussion

### Instrumental variables

Since 6-CINA has low volatility, it must be previously transformed into an adequate volatile compound prior to a GC analysis. For that, SPE extracts were derivatized prior to GC determination. Different derivatization agents were investigated and the chromatographic properties of various derivatives were studied. Methylation was performed by treating the eluent with methanol and concentrated H<sub>2</sub>SO<sub>4</sub>, followed by extraction with *n*-hexane.<sup>24</sup> It is a long, cumbersome and tedious process. Methyl derivatives were less sensitive to the mass spectrometer detector than the trimethylsilyl esters. They were obtained by a reaction of dried eluent with Sigmasil A.<sup>25</sup> Although trimethylsilyl derivatives were much more sensitive than methyl derivatives, the excess of the derivatizing agent and its by-products produced several interfering peaks. Hexafluoroisopropyl derivatives which are produced by a rapid coupling of the eluent with HFIP in the presence of DIC reaction, are highly sensitive and do not produce interfering substances.<sup>21</sup>

The retention times for IS and 6-CINA-HFIP derivatives were 4.24 and 5.53 min, respectively. Less than 6 min of analysis were required for adequate separation of the analyte and IS with the selected GC conditions.

MS-MS spectra were obtained by collision-induced dissociation (CID).<sup>26,27</sup> Non-resonant excitation was selected for the compounds. The isolated precursor ions were selected from the full scan spectra of the compounds by taking into consideration its *m/z* and relative abundance (both as high as possible) in order to improve sensitivity and selectivity. The excitation storage level, which is related with the trapping field that stabilizes the precursor ion, was selected for each analyte at the minimum value that allows the dissociation of the ion. The excitation time and CID excitation amplitude were optimized with the object of

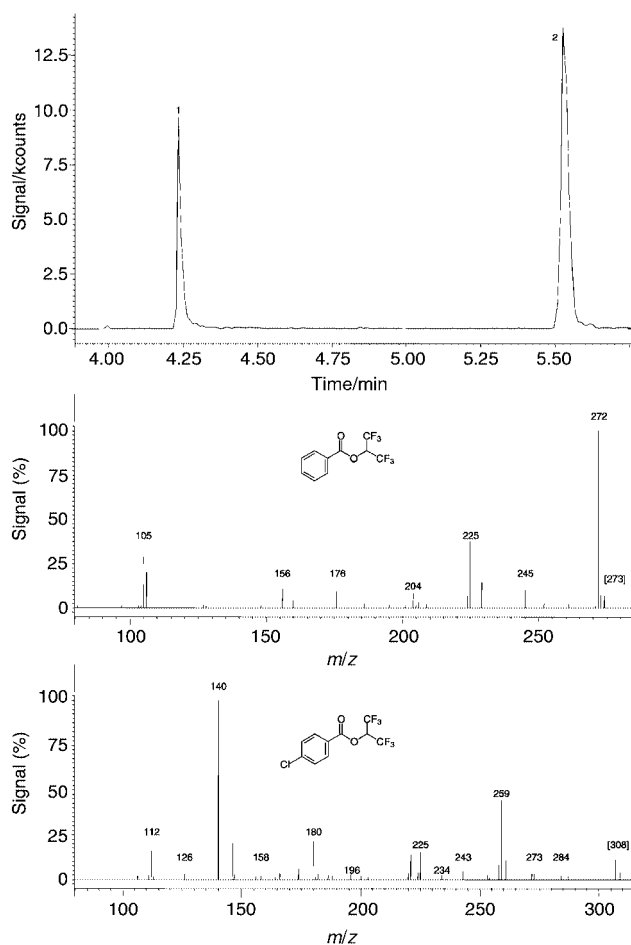
**Table 1** MS-MS conditions. (Excitation time = 40 ms; isolation window = 1 u; non-resonant waveform)

	IS	6-CINA
Activation time/min	4	5
Range ( <i>m/z</i> )	80–285	80–315
Precursor ion ( <i>m/z</i> )	272	308
Mass defect/ $\mu$ per 100 u	+47	-136
Excitation storage level ( <i>m/z</i> )	60	100
Excitation amplitude/V	40	82

generating CID spectra with the precursor ions as the molecular peak (between 10 and 20% of the relative abundance). The base peaks of the product ion spectra were used as quantification ions. The AGC target was fixed at 5000 counts because higher values caused electrostatic interactions between ions in the ion trap chamber. The EI-MS-MS spectra obtained for the IS and 6-CINA hexafluoroisopropyl derivatives using the selected conditions are shown in Fig 2. The breakages proposed for the main ions are summarized in Table 2.

## Sample preparation

On the basis of previous experiences it was decided to use SPE for sample preparation since it was found that eluents from the SPE cartridges were better suited for direct GC analysis. Liquid-liquid extraction involved worse recovery rates and



**Fig. 2** (a) Chromatogram of (1) IS and (2) 6-CINA in human urine at the fortification level of  $10 \text{ ng l}^{-1}$ . EI-MS-MS spectrum and structure of (b) IS-HFIP (precursor ion chosen 273) and (c) 6-CINA-HFIP (precursor ion chosen 308).

**Table 2** Important mass spectral fragments and their tentative ions

Compound	<i>m/z</i>	Tentative ion
IS-HFIP	273	[M] <sup>+</sup>
	272	[M-H] <sup>+</sup>
	245	[M-CN] <sup>+</sup>
	225	[M-CN-FH] <sup>+</sup>
	105	[C <sub>5</sub> H <sub>4</sub> N-CO] <sup>+</sup>
6-CINA-HFIP	308	[M] <sup>+</sup>
	259	[M-Cl] <sup>+</sup>
	225	[M-CN-FH-Cl] <sup>+</sup>
	180	[M-CF <sub>3</sub> -3F] <sup>+</sup>
	140	[Cl-C <sub>5</sub> H <sub>4</sub> N-CO] <sup>+</sup>
	112	[Cl-C <sub>5</sub> H <sub>4</sub> N] <sup>+</sup>

needed a clean-up step to avoid reduction in column efficiency and contamination of injector and ion trap.

Acidic hydrolysis of possible conjugates of 6-CINA was performed by adding sulfuric acid and heating. Thus free metabolites were obtained prior to the extraction. Amberlite XAD-4 was selected as sorbent after testing other materials (500 mg of C18). Generally, adsorbing of the analyte to the resin is allowed until adsorbate leakage in the column effluent increases to a predetermined level. At this point, the adsorbed compound is removed by elution.<sup>28</sup> For this reason, the amount of sorbent packed in the cartridges was studied. Different aliquots of clean urine containing  $100 \text{ ng mL}^{-1}$  of the metabolite were analyzed with cartridges filled with amounts of sorbent that ranged between 500 and 1000 mg. The results showed that less than 700 mg of XAD-4 may express saturation for the concentration studied (Fig. 3).

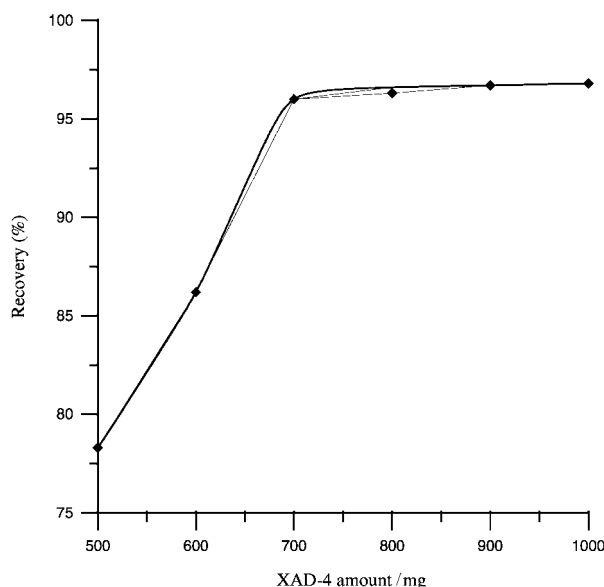
Several solvents (acetone, hexane, diethyl ether, methanol and dichloromethane) were tested for elution efficiency. The best elution results were found using 7 mL diethyl ether. A selective clean-up step was made by passing through the cartridge 2 ml of acidified water (pH = 1.3) and 2 ml of hexane. In this way, several interfering substances were removed from the extract without a reduction of the extraction efficiency.

## Influence of pH

The adsorption of carboxylic acids in a bed of Amberlite XAD-4 is strongly influenced by pH.<sup>29</sup> Generally, potentially ionic compounds such as carboxylic acids will be much more strongly adsorbed in the non-ionic form than in the ionic form. The influence of pH on the extraction process was studied determining the recovery rate of the extraction of different aliquots of clean urine spiked with  $100 \text{ ng mL}^{-1}$  of 6-CINA at pH values that range from 0 to 5.5. The results shown in Fig. 4 indicate that recoveries are close to 100% when pH is < 2.8. Nevertheless, a very high concentration of protons (pH < 0.8) negatively affected the resin adsorption efficiency (Fig. 4). Therefore, the pH of the urine sample must be between 0.8 and 2.8 before the sorbent extraction.

## Analytical parameters

GC-MS-MS analysis of working standard solutions consisting of the derivatives of relevant compounds was performed. The



**Fig. 3** Influence of the amount of XAD-4 resin on the extraction process.

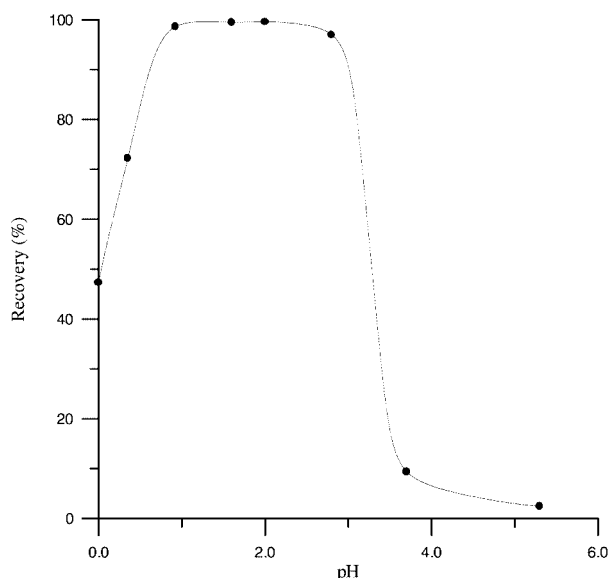


Fig. 4 Influence of pH on the extraction process.

Table 3 Analytical parameters

Intercept ( <i>a</i> )	-0.50
Slope ( <i>b</i> )	3.05
Correlation coefficient ( <i>r</i> <sup>2</sup> )	99.87
Lack of fit test ( <i>P</i> -value)	0.59
LOD/pg mL <sup>-1</sup>	16
LOQ/pg mL <sup>-1</sup>	56
Recovery (RSD)/10 ng mL <sup>-1</sup>	102.1% (5.4%)
Recovery (RSD)/100 ng mL <sup>-1</sup>	97.2% (3.5%)

resultant spectra were included in an EI-MS-MS spectra library. The target analyte was searched for by its retention time ( $\pm 10$  s window), and confirmed by comparison with the EI-MS-MS spectra in the library (minimum spectral fit required  $> 700$  and the signal-to-noise level  $S/N > 3$ ). With MS-MS, if a hypothetical co-eluted interference has the same ion used as precursor ion for our analyte, such an interference can be avoided using special experimental conditions for the CID and quantifying with a specific ion from the CID analyte spectrum.

The instrumental calibration was performed applying the proposed method to aliquots of a clean urine sample spiked with 6-CINA in a concentration range between 0.5 and 100 ng mL<sup>-1</sup> (IS, 100 ng mL<sup>-1</sup>). The calibration results are shown in Table 3. A lack-of-fit test<sup>30</sup> was carried out for three replicates of each concentration in order to check the linearity of the calibration graphs. The value found for the test (*P*-value) demonstrated good linearity in the studied range.

To determine detection (LOD) and quantification (LOQ) limits, urine without pesticides or metabolites was analyzed. The study of 10 blanks followed IUPAC recommendations.<sup>31</sup> LOD and LOQ calculated are in the low parts per trillion (ppt) level (Table 3). It is a good indication of the sensitivity of the technique employed (GC-MS-MS). High sensitivity, may in part, be attributed to the complete elimination of undesirable matrix background and the considerably high instrumental response of the HFIP esters.

The extraction efficiency of the proposed SPE method was assessed. Recovery and repeatability studies were carried out at the fortification levels of 10 and 100 ng mL<sup>-1</sup> ( $n = 10$ ). The results obtained are represented in Table 3. The mean recovery rates were between 97 and 102% and the repeatability, expressed as relative standard deviation (RSD %) was lower than 5.4% in all the cases. These precision results reveal, among other things, that the process of making the extraction cartridges can be considered routine.

## Applications

The proposed method was applied to the determination of the 6-CINA metabolite in urine samples of five agricultural workers from Almería (Spain) and the chromatograms obtained showed no peak for either the metabolite or the matrix interference. The analyses of laboratory reagent blanks and laboratory spiked matrix samples were performed together with the set of samples. Laboratory spiked urine samples eliminated any recovery deficiency during processing samples.

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