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## Development and validation of an assay of Phosmet residues in human blood by GC/MS

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### ABSTRACT

An assay of phosmet residues in human blood by gas chromatography coupled to mass spectrometry (GC/MS) Clarus® 600/560 DMS PerkinElmer® was developed and validated in human blood samples loaded by residues of this insecticide. The extraction of phosmet has been carried out in solid phase through C18 SPE cartridges. The proposed method of phosmet residue analysis by GC/MS uses a capillary type Supelco®, the oven temperature was programmed from 75°C to 320°C. The Helium was used as carrier gas with a flow rate of 0.8 ml / min. The Phosalone was used as internal standard (IS). The retention time of the analyte and EI are 14.34 and 14.67 ± 0.02 min, respectively. The calibration curve is linear in the concentration range between 10 and 100ppb with R2 = 0.998. The method was validated according to ICH guidelines Q2 (R1) in terms of the criteria of accuracy, precision, linearity and specificity. The validated method is selective and has a quantification limit of 10 ppb. It can be used routinely for the determination of phosmet residues in real samples of human blood.

**Keywords:** Validation, Phosmet residues, Human blood, Analysis, GC / MS.

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### INTRODUCTION

Phosmet (2-diméthoxyphosphinothioylthiométhy isoindoline-1.3-dione) (Fig 1), is an insecticide from the class of non-systemic organophosphate, used mainly to treat the apple trees to fight against codling moth. It is also used to treat many other fruit and ornamental crops. Its application on the vine is to fight especially against aphids, mites and fruit flies [1].

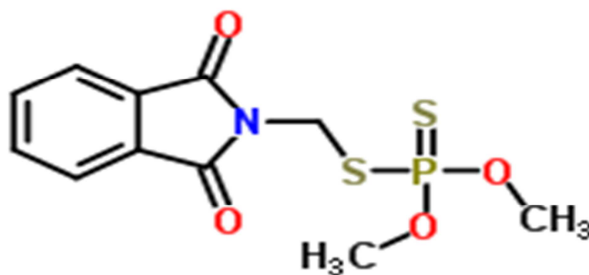


Figure 1: Chemical structure of Phosmet [2]

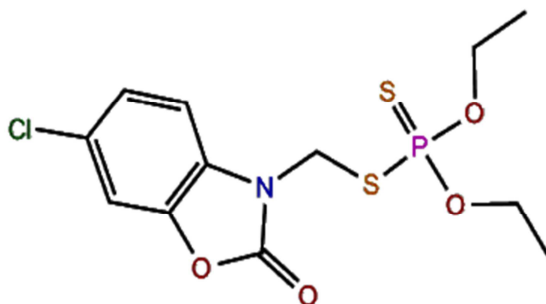


Figure 2: Chemical structure of Phosalone [3]

The main action of phosmet is typical of the class of organophosphate; it acts by inhibiting the enzyme acetylcholinesterase, with interruption of transmission of nerve impulses and subsequent accumulation of acetylcholine [4]. Symptoms of chronic poisoning phosmet are: the degeneration of nervous system cells by cumulative effects related to inhibition of cholinesterase, liver degeneration and decreased weight [5], diffuse brain atrophy [6] the decline in intellectual performance [7]. The assay technique volatile pesticides in the blood samples by GC/MS that was developed by Eric LACASSIE [8] has enabled the quantification of phosmet in the presence of other pesticides with cyproheptadine as internal standard. The pesticides are extracted from the blood in the solid phase on Oasis® types of cartridges and the chromatographic analysis are carried out by automatic injection of 2 µl of the final organic extract into the GC / MS system. Other pesticide assay of chromatographic techniques using the method of solid-phase micro extraction (SPME) allow the determination of phosmet residues in human blood such as that developed by Pawliszyn et al [9,10]. The presence of several pesticides in a single sample can make phosmet dosage by these multi residual nonspecific methods. So it seemed sensible to develop a new method for determination of phosmet residues in human blood by GC / MS. The main objective of this work is to quantify residues of phosmet by using of phosalone (Fig 2) as intern standard with high accuracy in the blood of people who are in direct contact (workers and farmers) or indirect (patients and consumers) with this residue, in order to prevent against the possible health complications in this population.

## MATERIALS AND METHODS

### Chemicals and reagents

Standard phosmet and phosalone (EI) are Scientique Ultra brand (USA, Kingstown). Acetonitrile, hexane, ethyl acetate and isooctane are solvents Ultrapure grade HPLC LiChrosolv Merck KGaA 64271 (Darmstadt, Germany). The standard solutions are diluted by HPLC grade methanol (VWR Prolabo, France). The SPEC18 extraction cartridges are brand (Waters USA). The sample of human blood free of traces of phosmet which has been used as a biological matrix during the development of the method comes from the blood transfusion center of Rabat, Morocco.

**Instrumentation**

The gas phase chromatographic system coupled to mass spectrometry is of type GC/MS Clarus® 600/560DMS PerkinElmer® (Bridgeport, USA), equipped with an automatic injector. The system is controlled by a mass Turbo Software (Windows XP SP2). The stationary phase is a column supelco® (L 30m x 0.25 ID x DF 0.25) Elite-5MS phase, the carrier gas is helium at a flow rate of 0.8 ml / min.

**Chromatographic conditions**

We have prepared a calibration range (10, 20, 30, 50 and 100 ppb) by serial dilution of the stock solution of phosmet (1 mg / mL) in plasma sample.

1 mL of each sample was mixed with 100 µL of a solution phosalone (EI) 100 ppb, the whole was mixed by vortexing for 20 seconds.

**Extraction**

The extraction was carried out by adding 1.5 mL of acetonitrile in a 15 mL conical tube containing 1ml of plasma. The mixture was stirred by vortex and centrifuge at 4000g for 10 min, the organic phase recovered is passed through a SPEC18 cartridge. Elution is made with an organic mixture (hexane / ethyl acetate 6/1 v / v). This organic phase is evaporated under a stream of nitrogen at a temperature of 50°C. The dry extract obtained is taken up in 100 µL of isooctane before injection of 1 µL into the chromatographic system.

**Oven Programming**

The oven was programmed from 75°C to 320°C at a gradient of 20°C per minute with a pre-heating the transfer line at 325°C and 250°C at source. The automatic injector in splitless is guide (50/1 to 25°C). Ionization is caused by electron impact (EI).

**RESULTS AND DISCUSSION**

Fig 3 shows chromatograms of phosmet and that phosalone which are retained in respective retentions times to (14.34 ± 0.02) and min (14.67 ± 0.02) min.

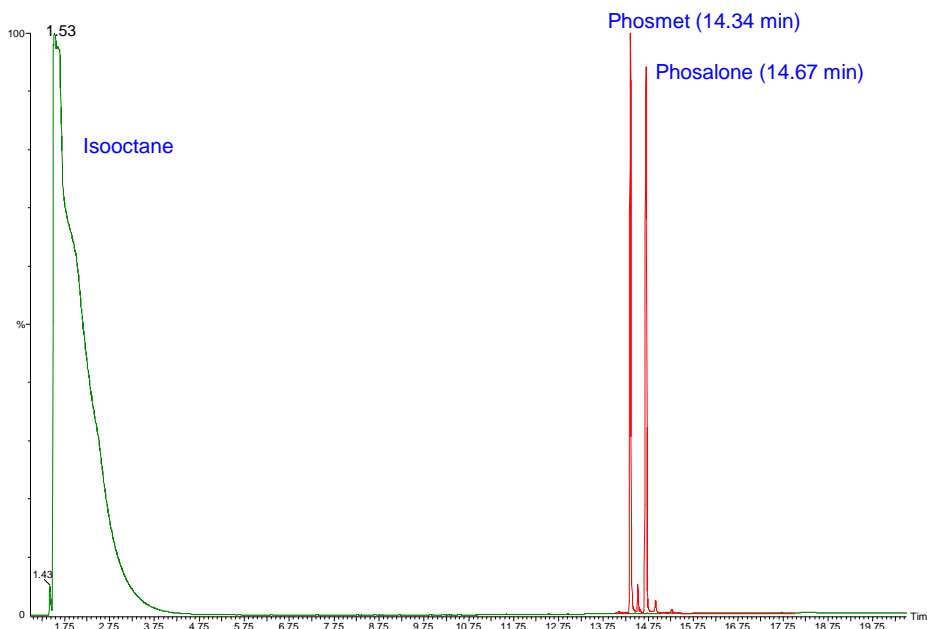


Figure 3: Chromatogrammes of phosmet (50ppb) and of phosalone (100 ppb)

The mass fragmentation by electron impact gave spectra characteristics masses namely: The Phosmet that is characterized by the masses (160, 161.77, 93 and 317 M / Z) and the phosalone that is identified by the mass (182, 77.97 and 367 M / Z) (Fig 4).

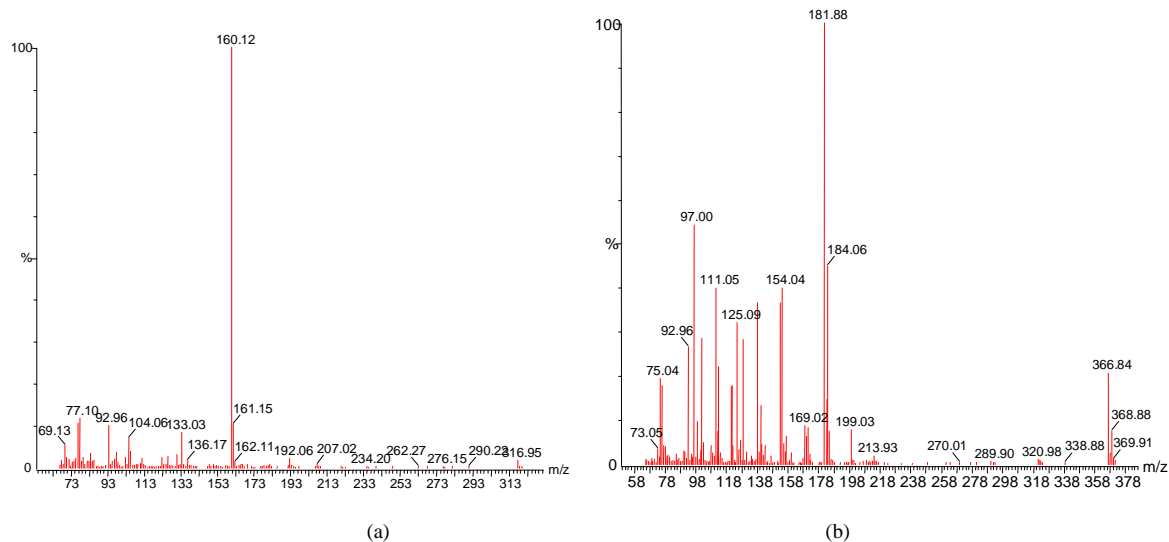
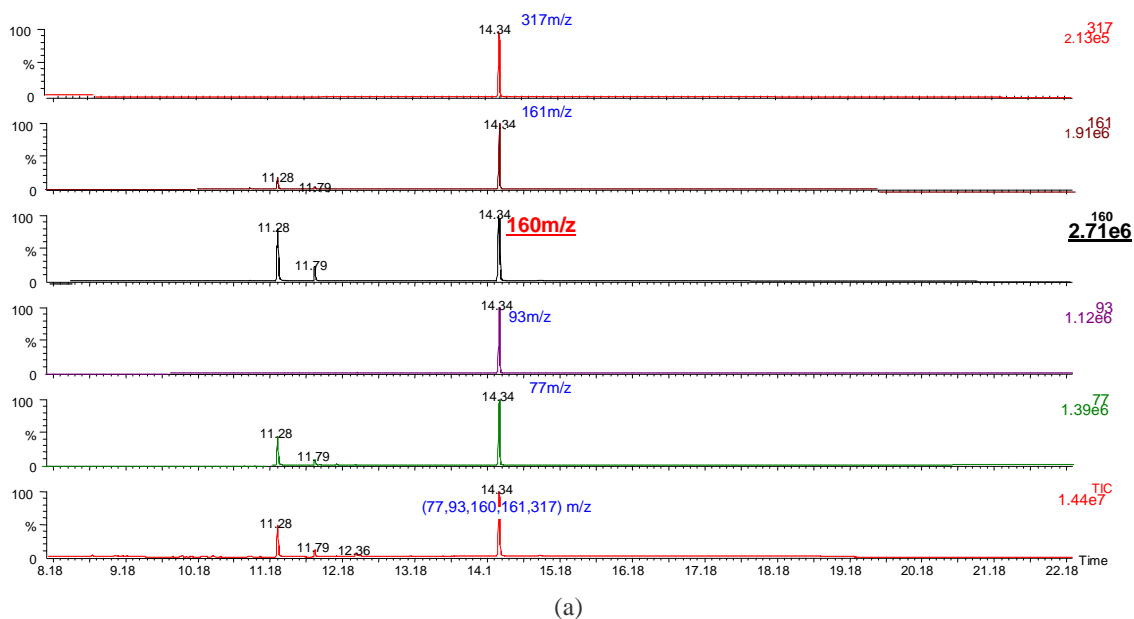
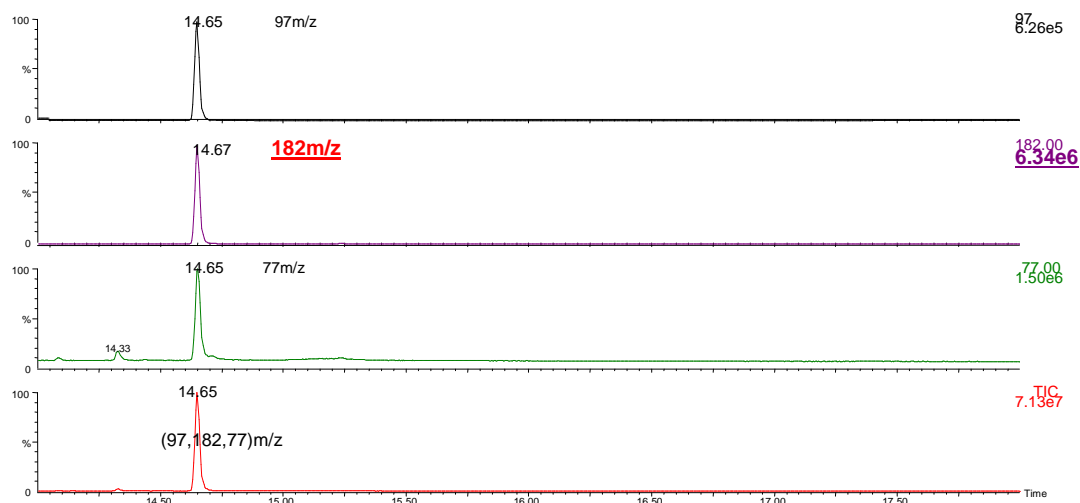


Figure 4: Mass spectra obtained by IE: (a) phosmet, (b) phosalone

The mass peaks 160 m / z for phosmet and 182 m / z for phosalone (Fig 5) which are the most abundant and the most stable were selected for development of the assay method.



(a)



(b)

**Figure 5: Selection of mass peaks for the assay: (a) Phosmet, (b) Phosalone**

The yield of absolute extract was calculated by choosing three points of the calibration range of 10ppb, 30ppb and 50ppb. An average yield of 86% **Table-1** confirms the reliability of extraction

**Table 1: extraction efficiency**

Concentrations	RA direct	RA (after extraction)	yield %
10 ppb	0.418	0.351	83.9
30 ppb	1.338	1.108	82.81
50 ppb	2.159	1.970	91.24
Yield moy %			<b>85.98</b>

### VALIDATION OF THE METHOD

The Validation is a crucial stage in the life cycle of an analytical method as it was highly regulated by different main repositories such as: NF EN ISO / IEC 17025 [11], Decisions 2002 / 657EC [12] the FDA recommendations [13] and ICHQ2 documents (R1) [14]. However, the validation criteria and methodologies have been widely defined and discussed in the literature [15,16], this made the validation become an essential aspect of modern analysis. [17] Among the criteria to be evaluated during the validation of an analytical method is cited; selectivity, linearity, accuracy, accuracy, limit of detection (LOD) and the limit of quantification (LOQ).

#### Selectivity

Selectivity was studied according to the validation standard [18]. The extracted white from the extraction of a blood sample free of phosmet and phosalone showed no signal at their respective retention times ( $14.34 \pm 0.02$ ) and min ( $14.67 \pm 0.02$ ) min (Fig 6).

#### Linearity

We verified the domain of the dosage and deduced the characteristics of the calibration line [19].

Over a dosing interval consisting of five concentration levels (10 ppb, 20 ppb, 30 ppb, 50 ppb and 100 ppb), each concentration was repeated 5 times.

The equation of the calibration straight line ( $y = 0.038x - 0.018$ ) was calculated by the least squares method on the whole range, with a correlation coefficient  $R^2 = 0.998$  (Fig 6).

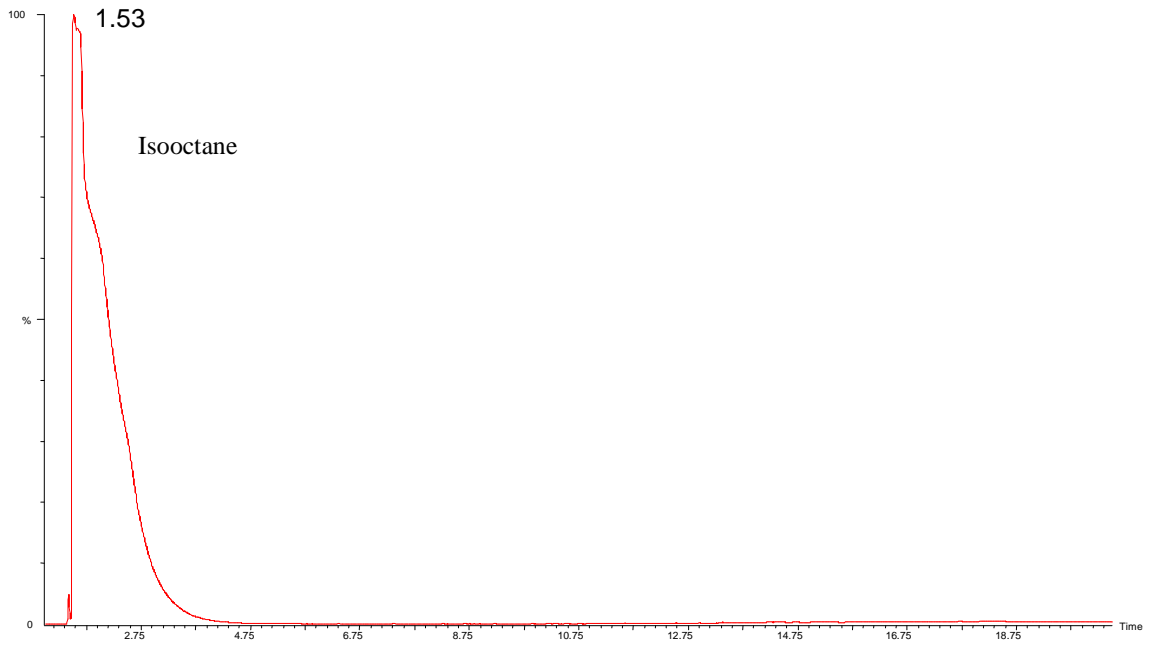


Figure 6: Chromatogram of selectivity

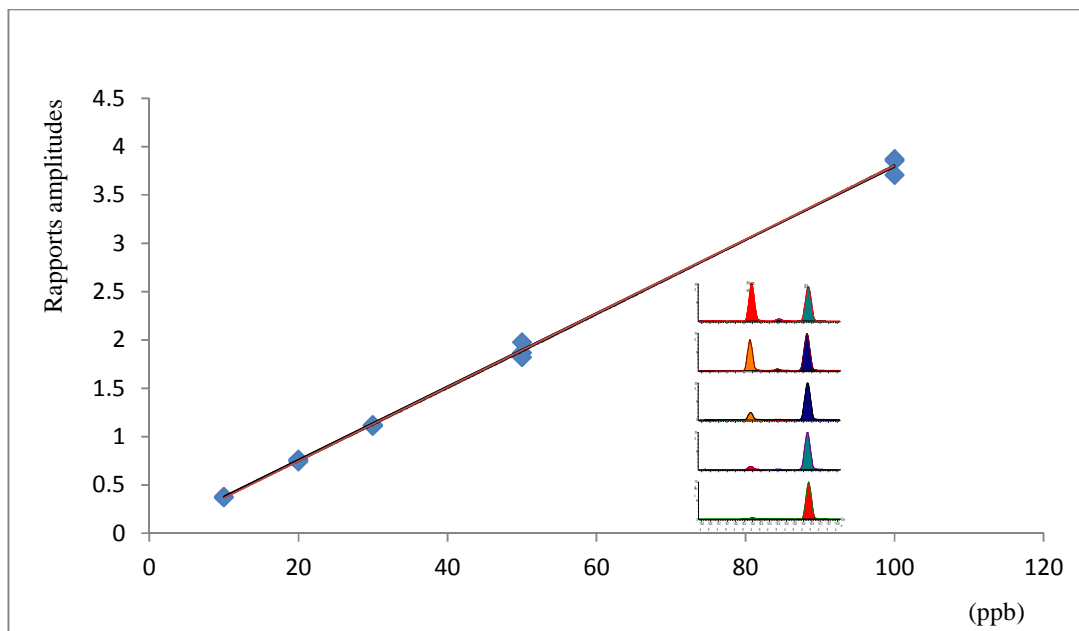


Figure 7: Right calibration phosmet

**Loyalty**

Faithfulness or precision is the degree of agreement between the results of measurements obtained by individual analysis of several samples of the same sample. The loyalty is expressed at two levels; repeatability and intermediate precision [20].

**Repeatability**

Is evaluated by the coefficient of variation (CVr or RSD) [20].

RSD% or CVr = standard deviation / mean x 100.

The repeatability of the method was studied on three levels of concentrations (20 ppb, 30 ppb and 50 ppb), these concentrations were repeated 5 times. CV computed is equal to 4.91%. **Table-2** indicating that the method is repeatable.

**Table 2: Results of repeatability**

Concentrations PPB	Quantites (n=5)	% recouvrement
20	20.456	102.28
30	34.350	114.50
50	55.826	111.65
<b>CVr</b>		<b>4.91%</b>

### Intermediate precision

This study was performed on three independent series at different concentration levels of 10 ppb, 20 ppb and 30 ppb. The coefficient of variation (CVR%) 8.2% **Table-3** is less than 15% recommended by the FDA in biological environment confirms the precision of the method.

**Table 3: Results of intermediate precision**

Concentrations PPB	Serie1	Serie2	Serie3
10	9.948	9.9053	9.766
20	19.500	22.053	19.218
30	26.632	35.026	27.789
<b>CVR</b>			<b>8.2%</b>

### Accuracy

It is the closeness of agreement between a test result and the accepted reference value by involving a combination of random components.

The average percentage recovery of the 5 points of the calibration range (10 ppb, 20 ppb, 30 ppb, 50 ppb and 100 ppb) is included in the 95% confidence interval ( $[m + t * S / \sqrt{100} \text{ 092 and } N = m - t * S / \sqrt{N} = 97,693]$ ). This range includes the 100% which shows the accuracy of the method.

### Limit of detection and quantification

For residues limit of detection (LOD) is estimated from the background noise of the recording,  $[20] \text{ LOD} = 3h_{\text{max}}$  ( $h_{\text{max}}$ : maximum amplitude of the signal over a distance equal to 20 times the width at half height of the peak corresponds to the substance to be searched).

The limit of quantitation ( $\text{LOQ} = 10h_{\text{max}}$ ) is the smallest amount of a substance to be examined in a sample that can be assayed in the experimental conditions described with fidelity and accuracy defined [20].

The LOD was determined to be 5 ppb (environ five times the background) and the LOQ was 10 ppb, hence the validity of this method for determination of phosmet residues is between 10 ppb and 100 ppb.

## CONCLUSION

The method of determination of residues of Phosmet developed from blood samples loaded using GC / MS as a technique, proves to be sufficiently sensitive, faithful, specific and accurate. It may be used successfully for the quantification of the phosmet in the human blood samples from persons deemed infected.

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