

Reprint from

**10th Cologne Workshop On Dope Analysis
7th to 12th June 1992
- Proceedings -**

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Sport und Buch Strauß, Köln, 1993

R. VENTURA, T. NADAL, M.J. PRETEL, A. SOLANS, J.A. PASCUAL, J. SEGURA:
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In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) 10th Cologne
Workshop On Dope Analysis 7th to 12th June 1992. Sport und Buch Strauß, Köln, (1993) 231-248

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Possibilities of Liquid Chromatography-Mass Spectrometry for the Identification of Labile Compounds

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Introduction

Gas chromatography/mass spectrometry (GC/MS) has been widely used for the identification and quantitation of drugs and their metabolites in biological samples. The widespread use of GC/MS is mainly due to the existence of very sensitive and easy-to-use commercial GC/MS instrumentation, powerful automation software, and large data base of reference electron impact ionization mass spectra.

However, many drugs and their metabolic products are not directly amenable to GC/MS analysis, because of their thermal lability or low volatility. Chemical derivatization can often overcome these problems, although there are many compounds which are not easy to be quantitatively converted to volatile derivatives.

Liquid chromatography (LC) does not require vaporization of the sample and therefore is fundamentally suited for the separation of thermally labile and low volatile analytes. Due to the great development on instrumentation in the last decade, LC/MS has become one of the most powerful analytical techniques for the analysis of such compounds in complex mixtures.

In this paper, LC/MS with a particle-beam interface is applied to the detection of thermolabile compounds of doping interest, such as morazone and mesocarb.

Experimental

Instrumental analysis

Liquid Chromatography/Ultraviolet detection:

LC/UV analyses were performed with a Hewlett-Packard 1090 liquid chromatograph with a diode array detector. Instrumental conditions were those described by Ventura et al (1).

Liquid Chromatography/Mass Spectrometry:

LC/MS analyses were performed with a Hewlett-Packard 5989 mass spectrometer coupled to a 59980B particle-beam interface, and a 1090L liquid chromatograph. The operating parameters of the interface were: desolvation chamber temperature, 70°C; helium pressure, 50 psi; nebulizer, -1.

Liquid chromatographic column was an Ultrasphere ODS, 25x0.2 cm, 5 μ m (Beckmann), and the mobile phase flow-rate was 0.4 ml/min.

The mobile phase composition for morazone analyses was 70% deionized water (with 0.5% of formic acid) and 30% acetonitrile. For mesocarb analyses, two mobile phases were used: 50% 0.1M ammonium acetate solution (with 0.5% of formic acid) and 50% acetonitrile, to analyze mesocarb methanolic solutions; and 62% 0.1M ammonium acetate solution (with 0.5% of formic acid) and 38% acetonitrile, to analyze urine extracts.

Electron impact ionization (70 eV) and SCAN acquisition were used in all analyses (m/z 50-390, for morazone analyses; and m/z 73-550, for mesocarb analyses).

Gas Chromatography/Mass Spectrometry:

GC/MS analyses were performed with a Hewlett-Packard 5890 gas chromatograph coupled to a 5971 mass selective detector. The injection port and the detector temperatures were 280°C.

For the analysis of morazone the column was a 5% phenylmethyl silicone (12.5 m, 0.2 mm, 0.33 μ m), and the temperature program was from 90°C to 280°C at 20°C/min. The final time was 5 min. Analyses were performed in SCAN mode (m/z 40-400).

For the analysis of mesocarb the column was a 5% phenylmethyl silicone (25 m, 0.2 mm, 0.33 μm), and the temperature program was from 100°C to 290°C at 20°C/min. The final time was 9.5 min. Analyses were performed in SCAN mode (m/z 50-600).

Sample preparation

Extraction procedures:

For LC/MS analyses of mesocarb and all LC/UV analyses, samples (5 ml) were extracted at pH 9 with ethyl acetate (2 x 6 ml), using sodium chloride to promote the salting-out effect, following the procedure described by Ventura et al (1). The combined organic layers were evaporated to dryness and redissolved with a mixture of acetonitrile and water (200 μl). The injection volumes were 10 μl and 20 μl for LC/MS and LC/UV analyses, respectively.

For the GC/MS analyses of mesocarb, samples (5 ml) were hydrolysed (acidic hydrolysis, see below) and extracted at acidic pH with diethylether (5 ml). The organic layer was discarded, and the aqueous phase was adjusted to pH 9.6 and extracted with diethylether (5 ml) using anhydrous sodium sulphate. The organic layer was evaporated to dryness and redissolved in 50 μl of methanol. The injection volume was 2 μl .

For the LC/MS analyses of morazone, samples (5 ml) were extracted at strong alkaline pH with diethyl ether (2 ml) using anhydrous sodium sulphate. The whole organic layer was evaporated to dryness and redissolved with 100 μl of methanol. The injection volume was 10 μl .

Acidic hydrolysis:

To 5 ml of the sample, 1 ml of 6N hydrochloric acid and 100 mg of cysteine were added. The sample was heated for 1 h at 100°C.

Results and discussion

1. Analysis of morazone

Morazone is included in the list of banned substances with stimulant activity. In conventional GC conditions, problems of decomposition of morazone in the injection port have been described (2). Morazone is degraded to phenmetrazine and other compound (Figure 1).

Morazone is well analyzed by LC using UV detection (Figure 2), and shows a characteristic UV spectrum, that makes it easy its identification in urine extracts (Figure 3).

In doping control, confirmation analyses must be done by MS. Due to the degradation of morazone in conventional GC/MS conditions, LC/MS could be one method of choice for confirmation purposes.

After LC/MS analysis using a particle-beam interface, morazone shows one peak with a mass spectrum identical to that reported by Pflieger et al (3) (Figure 4). No larger differences are observed when changing the source temperature from 200 to 250°C. Mass spectra is similar, although a weak molecular ion (m/z 377) appears at lower temperature. In scan mode and 250°C of source temperature the minimum detectable quantity of morazone is 100 ng.

Using alkaline extraction with diethylether and LC/MS analysis, morazone is detected in urine extracts (Figure 5). Thus, LC/MS allows the direct confirmation of morazone ingestion.

2. Analysis of mesocarb and mesocarb metabolites

Mesocarb is a compound with stimulant activity recently introduced in the list of banned compounds in sport. Information concerning the human metabolism and urinary excretion of this product is limited. Hydroxylated metabolites free and conjugated are the main products described in rat urine (4, 5).

The analysis of these compounds is difficult due to their thermal lability. After GC analysis of methanolic solutions of mesocarb or hydrolysed urine extracts, the pyrolysis product common to mesocarb and their p-hydroxy metabolite is obtained (Figure 6).

Mesocarb is well analyzed by LC using UV detection and shows a characteristic UV spectrum (Figure 7). The analysis by LC/UV of urines obtained after mesocarb intake, shows a peak with shorter retention time than mesocarb and identical UV spectrum (Figure 8). No unchanged mesocarb is detected.

If the sample is hydrolysed prior to the extraction, this peak disappears and another peak with slightly larger retention time and the same UV spectrum appears (Figure 9). Unchanged mesocarb is not detected either.

After LC/MS analysis of mesocarb standard using electron impact ionization and a source temperature of 250°C (Figure 10), a narrow peak is obtained with a mass spectrum identical to that obtained by Tamás et al (4) and Polgár et al (5) using direct introduction of the compound into the MS. At lower source temperatures broad peaks are obtained indicating poor vaporization of the compound in the ion source (Figure 10).

The analysis by LC/MS of non hydrolysed urine extracts (Figure 11) gives a peak with mass spectrum related to that of the p-hydroxy-mesocarb previously described in rat urine (4,5). The mass spectrum shows a characteristic peak at m/z 135, indicating p-hydroxylation of the molecule, but the relative abundance between m/z 91 and 135 doesn't fit with that of p-hydroxy-mesocarb (4,5).

When hydrolysed urine extracts are analyzed by LC/MS, a peak showing a mass spectrum corresponding to p-hydroxy-mesocarb is detected (Figure 12). Additionally to the ions described (4,5) a low molecular ion (m/z 338) is also observed in our case.

The results obtained in this work indicate that a conjugated p-hydroxymesocarb is the main mesocarb metabolite present in human urine. Free p-hydroxymesocarb and unchanged mesocarb were not detected in the studied urines.

Conclusions

The usefulness of LC/MS for the analysis of labile compounds of doping interest present in complex mixtures (i.e. urine extracts) has been demonstrated. LC/MS with a particle-beam interface gives effective confirmation methods for morazone and mesocarb ingestion.

Acknowledgements

Technical assistance of P. Alcalde, M. Salmerón, M. Carnicero and R. Masagué is gratefully acknowledged.

References

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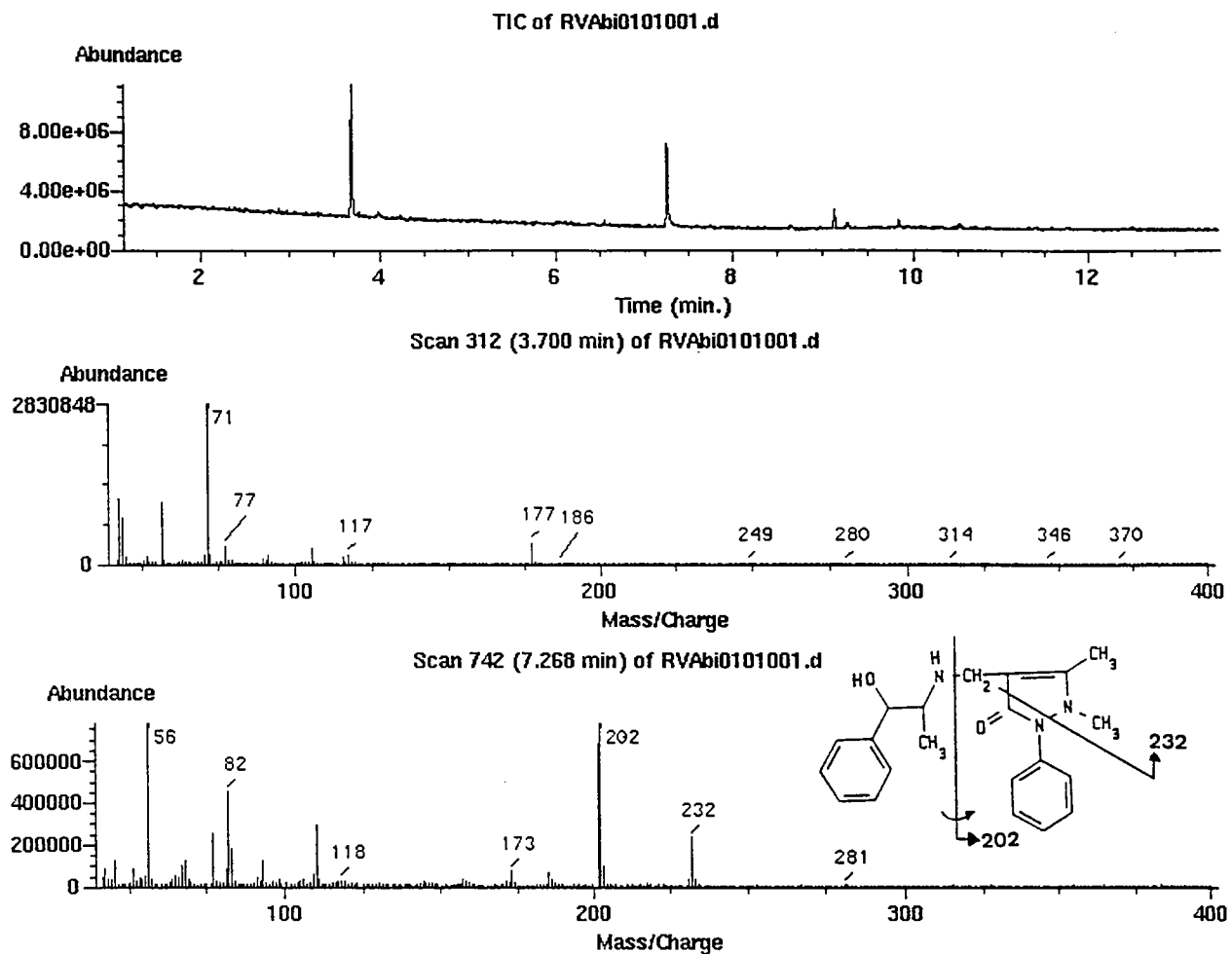


Figure 1. GC/MS analysis of a methanolic solution of morazone. TIC and mass spectra of the peaks detected: phenmetrazine (3.7 min) and unknown compound (7.2 min). A tentative structure for this compound is proposed.

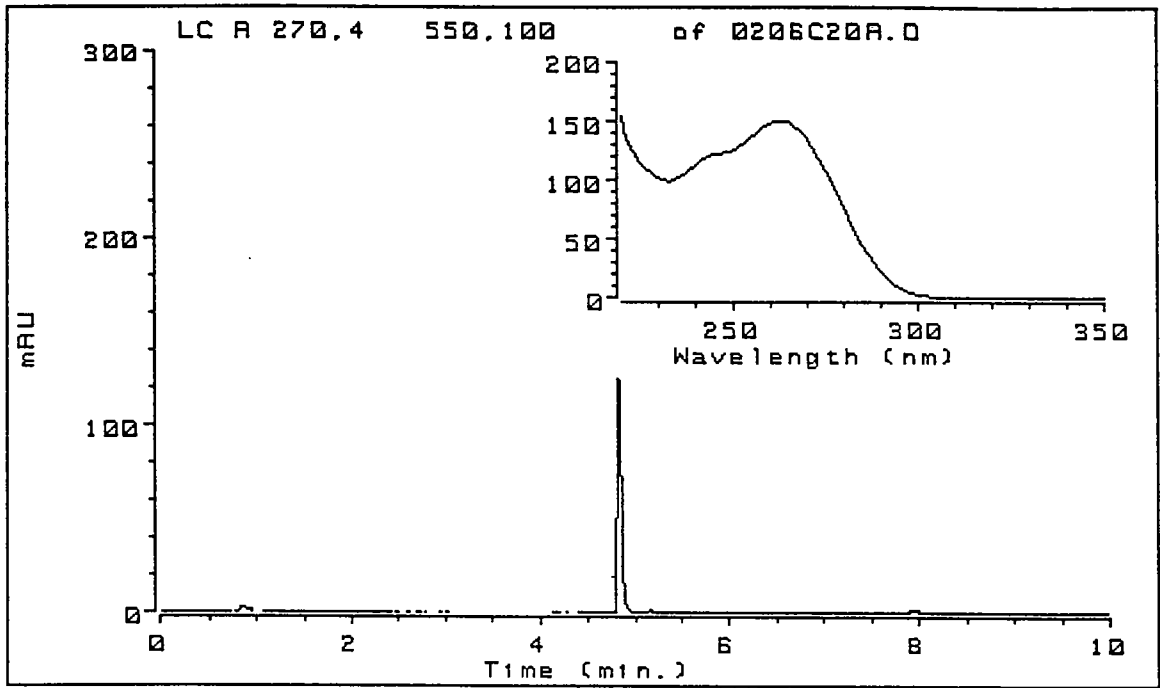
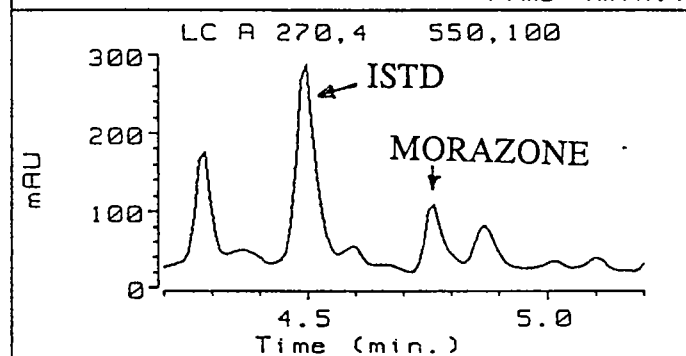
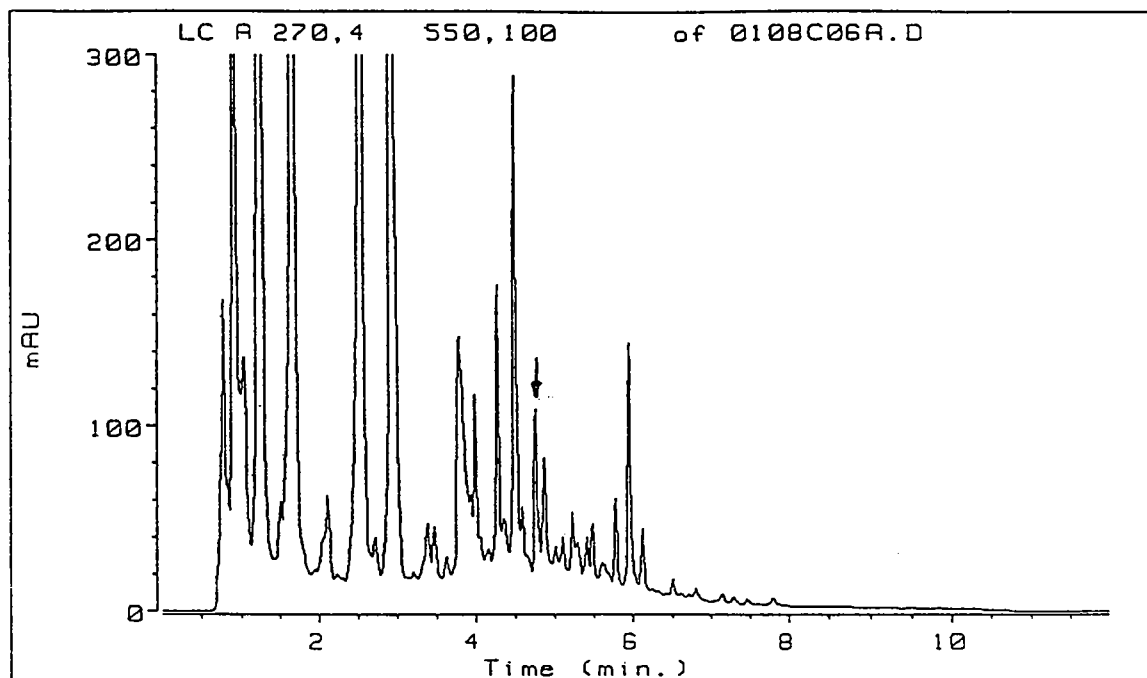
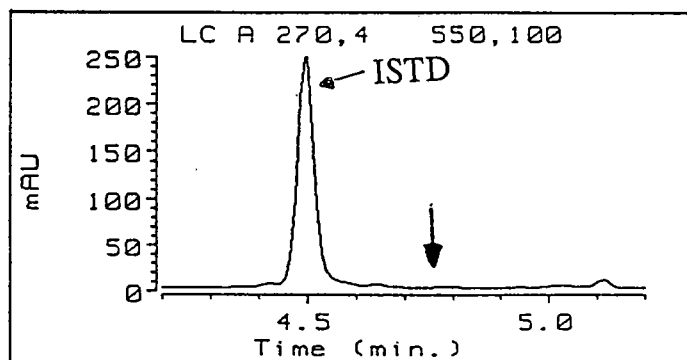
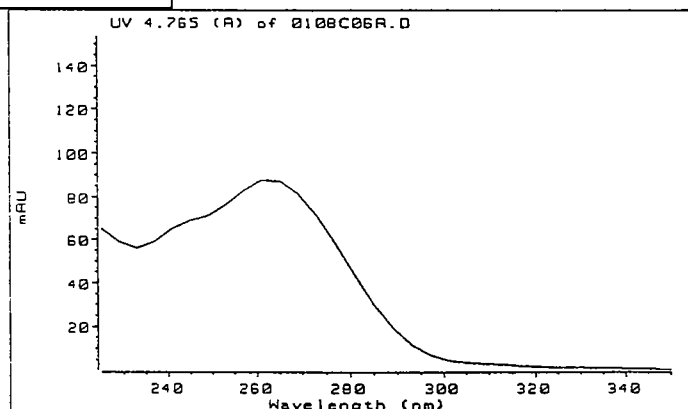


Figure 2. LC/UV analysis of a methanolic solution of morazone. Chromatogram at 270 nm and UV spectrum of morazone.



URINE POSITIVE TO MORAZONE



BLANK URINE

Figure 3. LC/UV analysis of a urine from an excretion study of morazone (chromatograms at 270 nm and UV spectrum of the peak of morazone) and a blank urine (chromatogram at 270 nm).

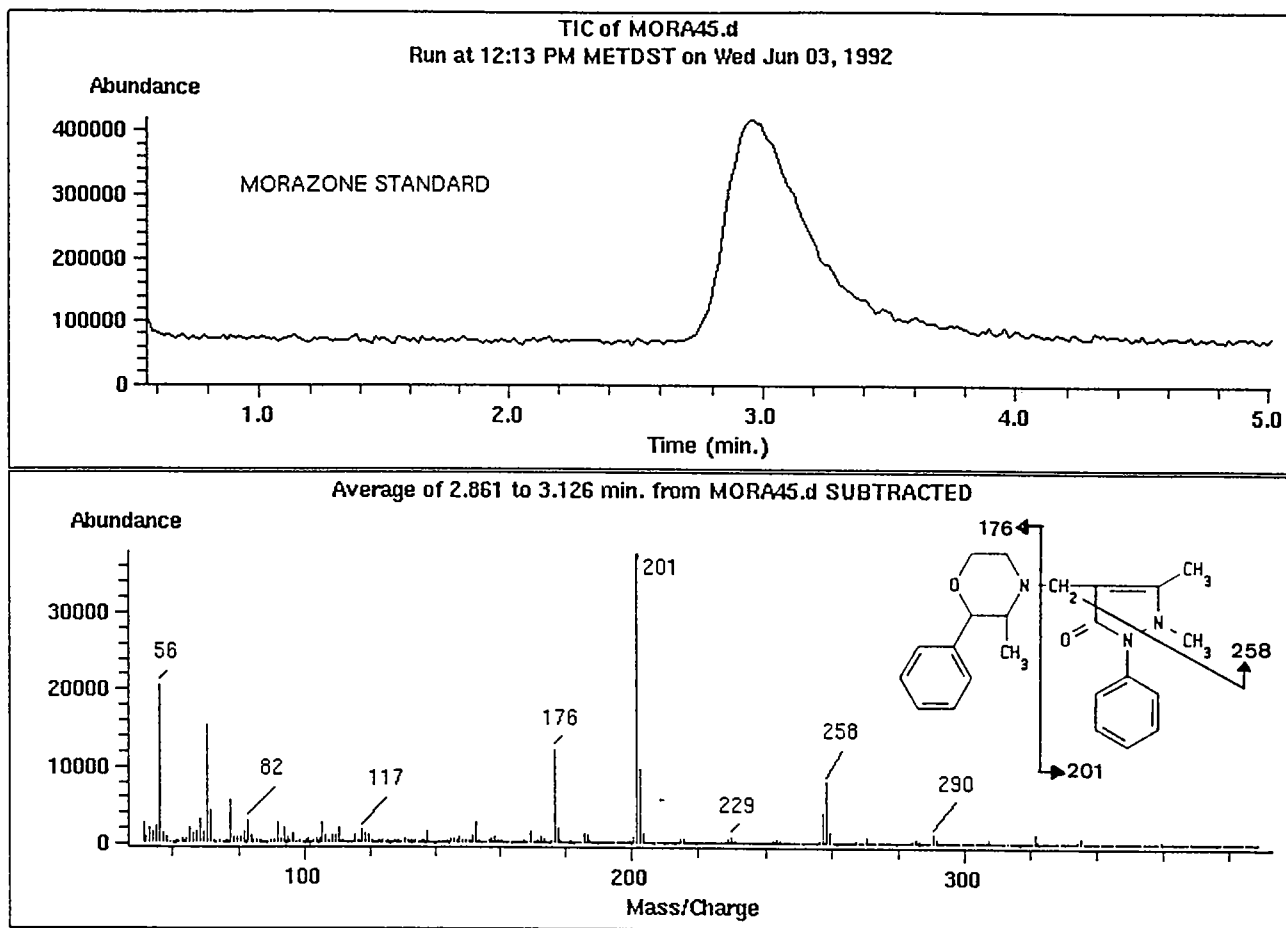


Figure 4. LC/MS analysis of a methanolic solution of morazone. TIC and mass spectrum of morazone.

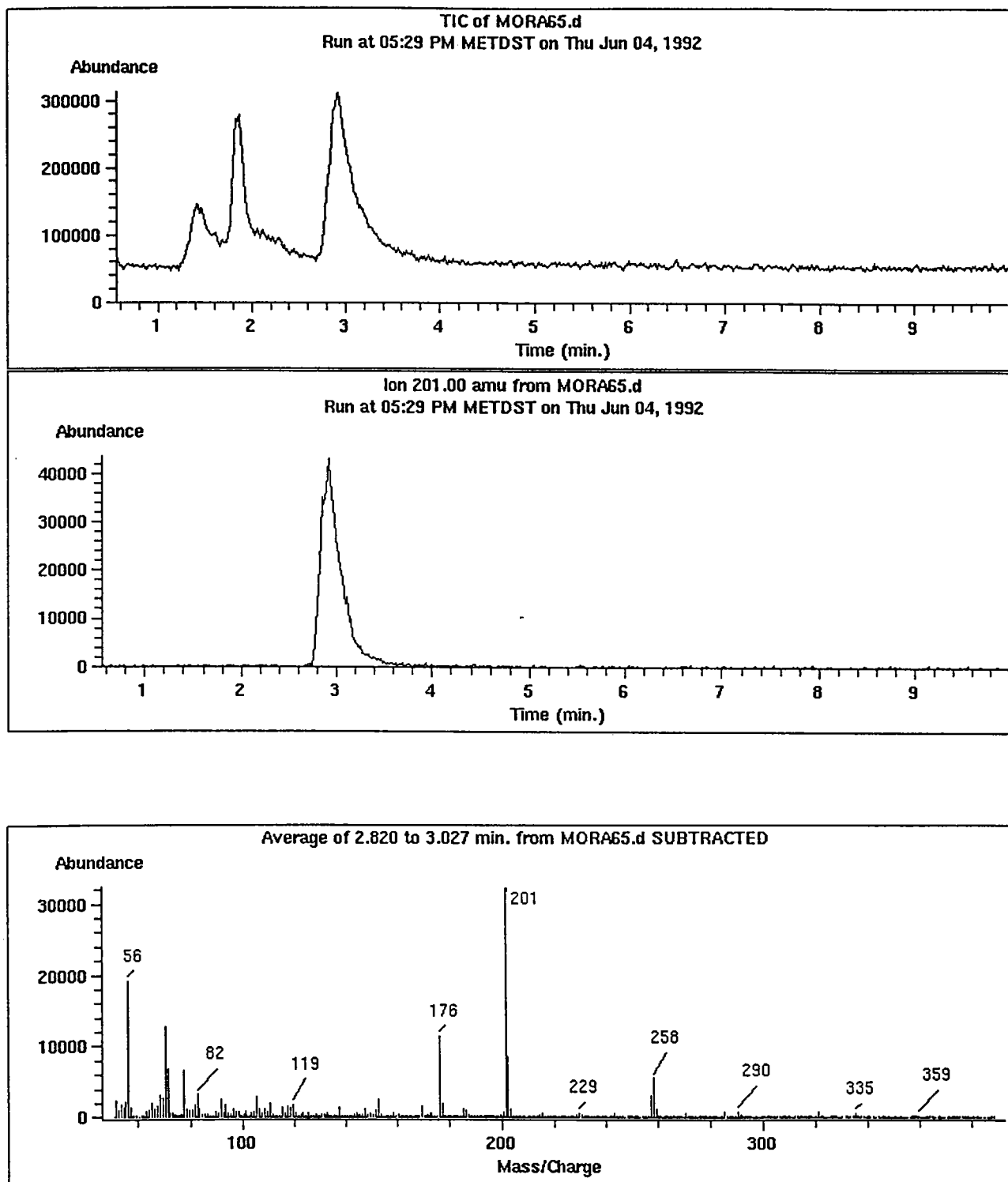


Figure 5. LC/MS analysis of a urine spiked with 2 $\mu\text{g}/\text{ml}$ of morazone. TIC, chromatogram of m/z 201 and mass spectrum of the peak detected.

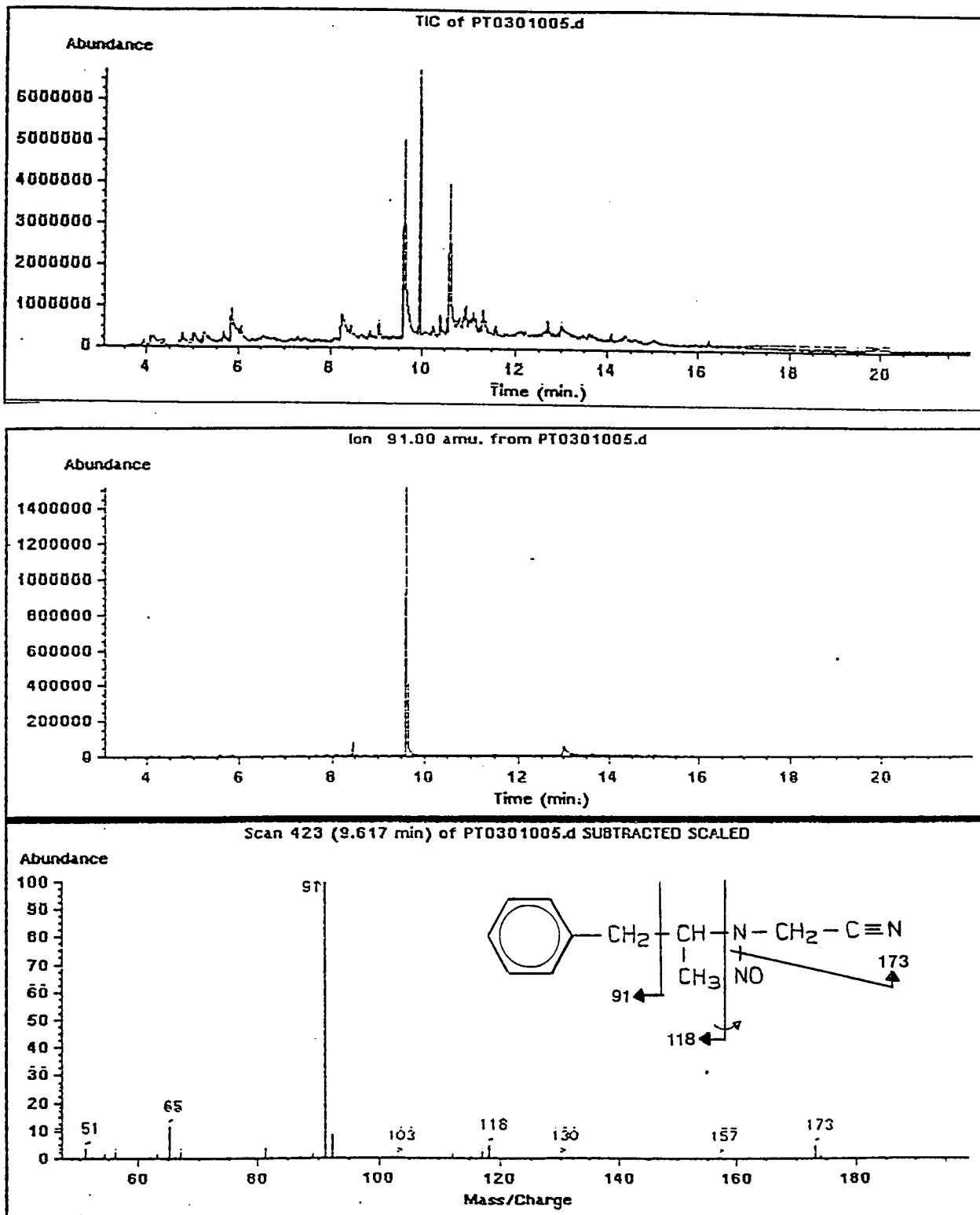


Figure 6. Chromatograms obtained after GC/MS analysis of a urine from an excretion study of mesocarb (TIC and chromatogram of m/z 91), and mass spectrum of the peak detected.

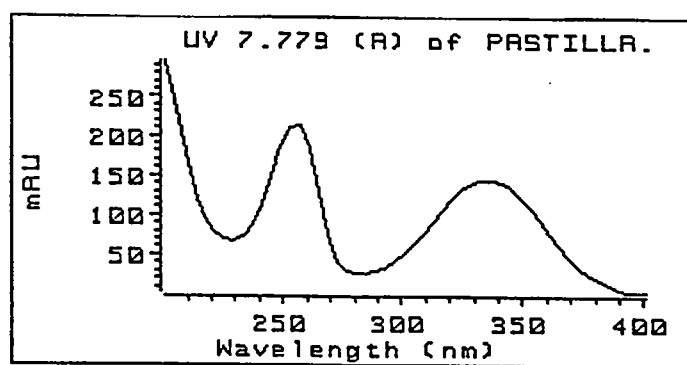
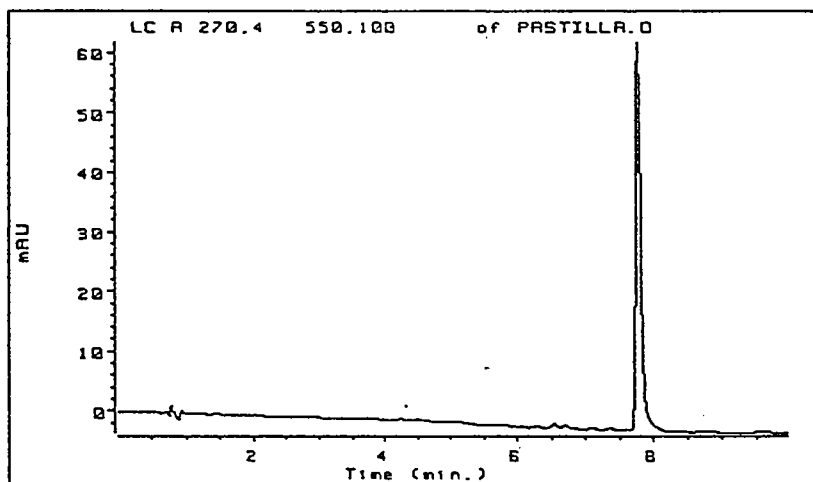
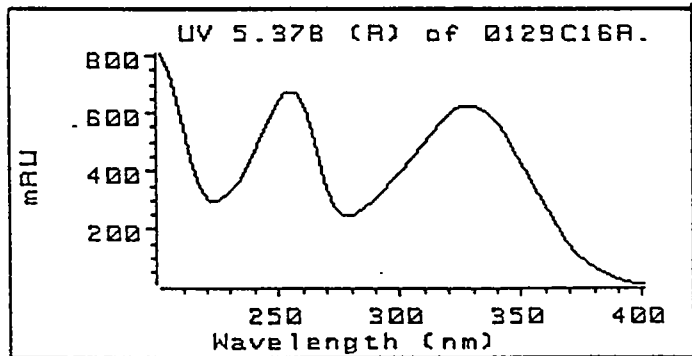
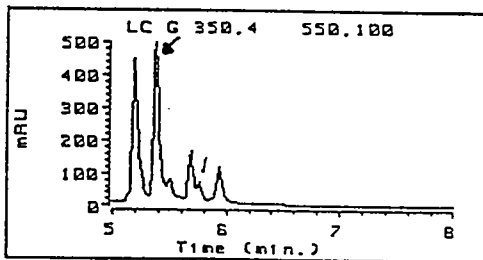
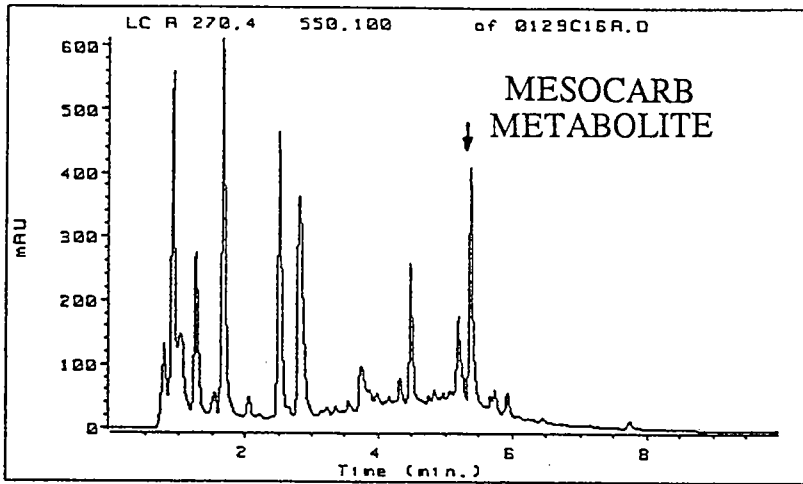


Figure 7. Analysis of a methanolic solution of mesocarb by LC/UV and characteristic UV spectrum of mesocarb.

POSITIVE URINE



BLANK URINE

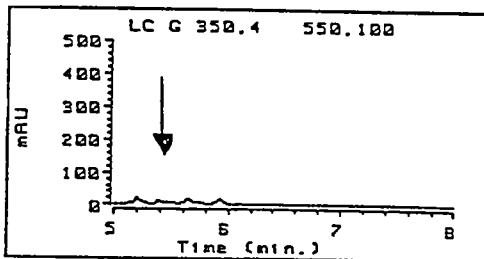
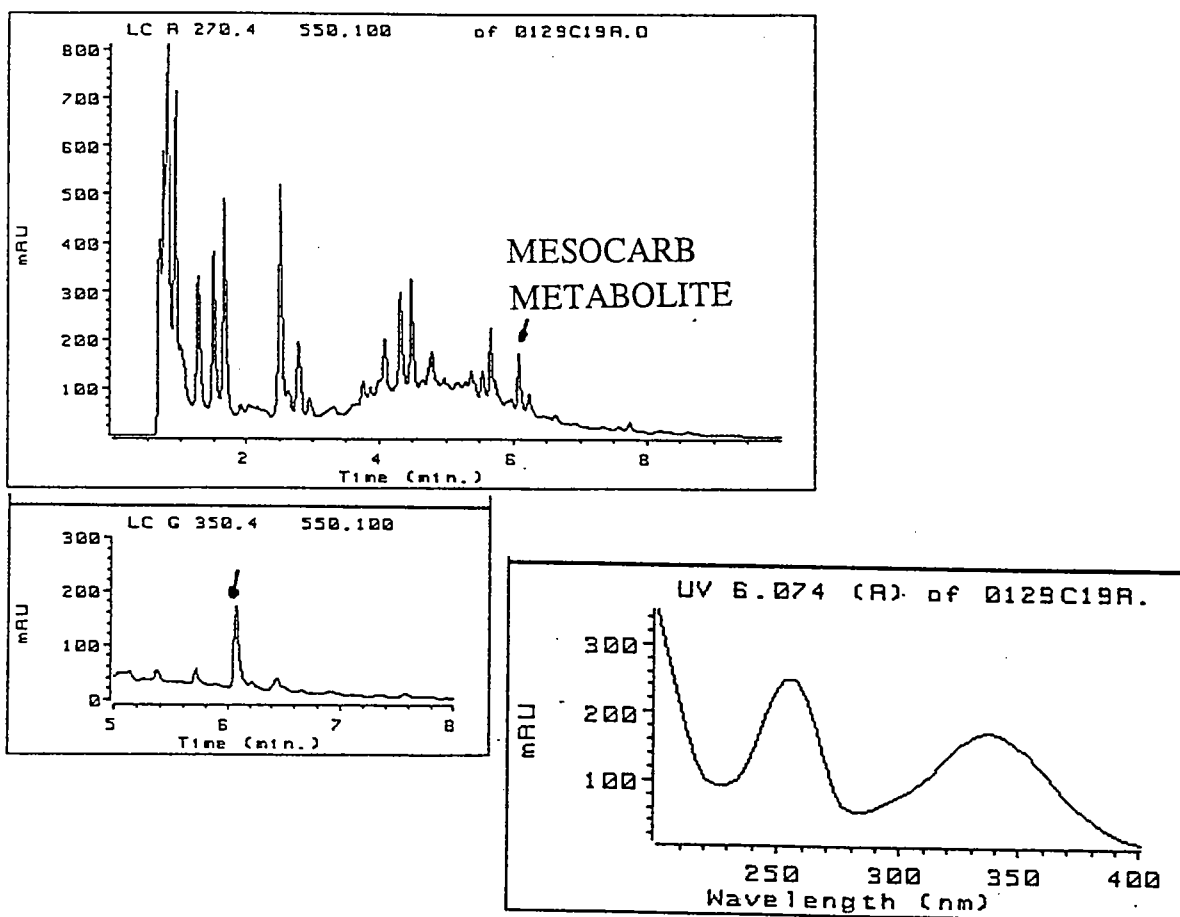


Figure 8. Chromatograms at 270 and 350 nm, and UV spectrum of the peak detected after analysis by LC/UV of a non hydrolysed urine extract from an excretion study of mesocarb, and chromatogram at 350 nm of a blank urine.

HYDROLYSED POSITIVE URINE



HYDROLYSED BLANK URINE

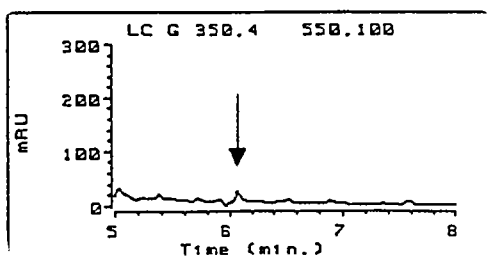


Figure 9. Chromatograms at 270 and 350 nm, and UV spectrum of the peak detected after analysis by LC/UV of an hydrolysed urine extract from an excretion study of mesocarb, and chromatogram at 350 nm of an hydrolysed blank urine.

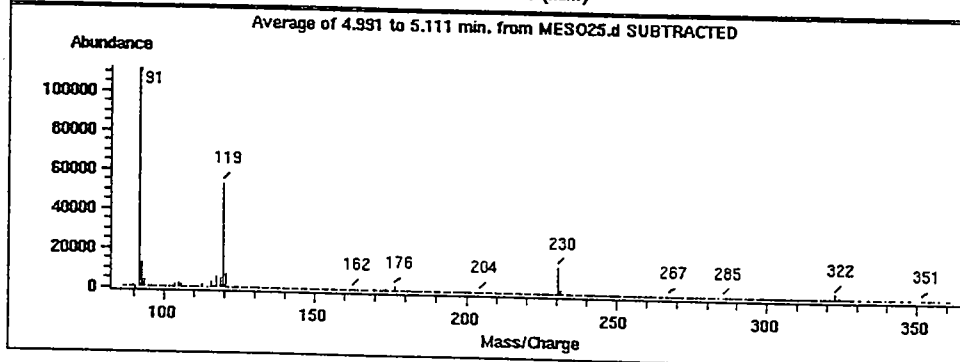
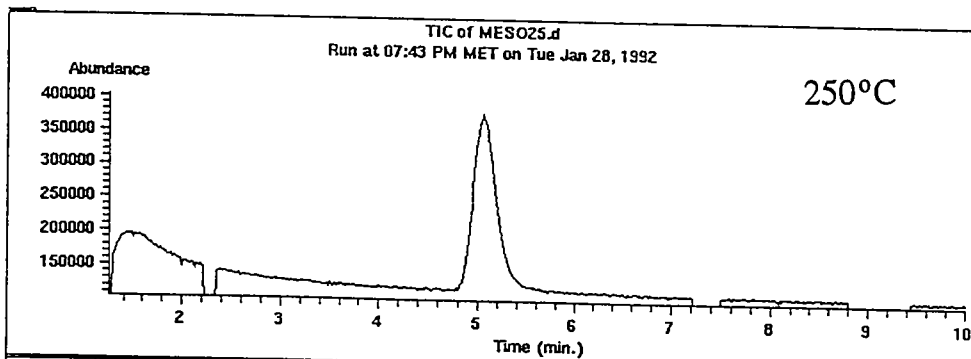
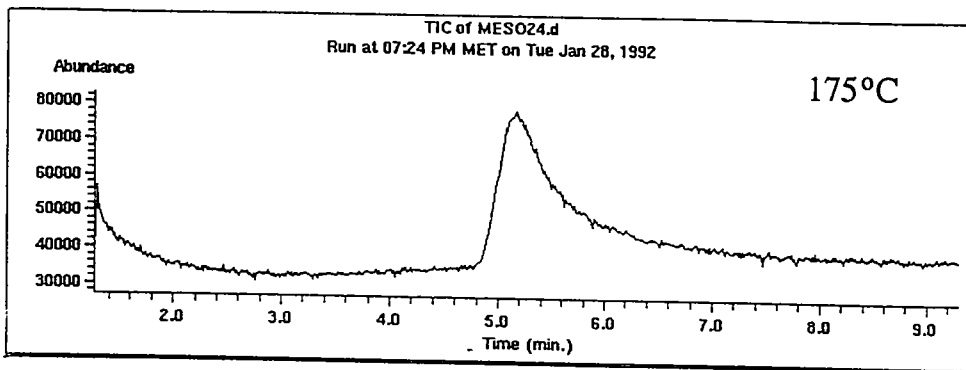
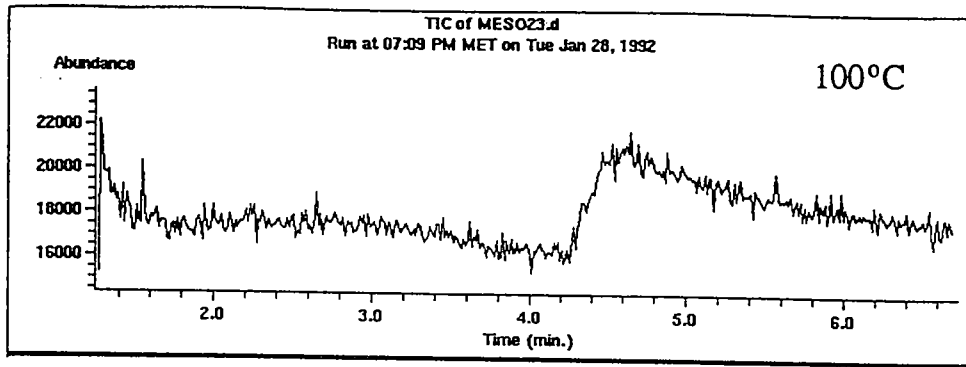


Figure 10. Analysis of a methanolic solution of mesocarb at different source temperatures, and mass spectrum of the peak obtained at 250°C.

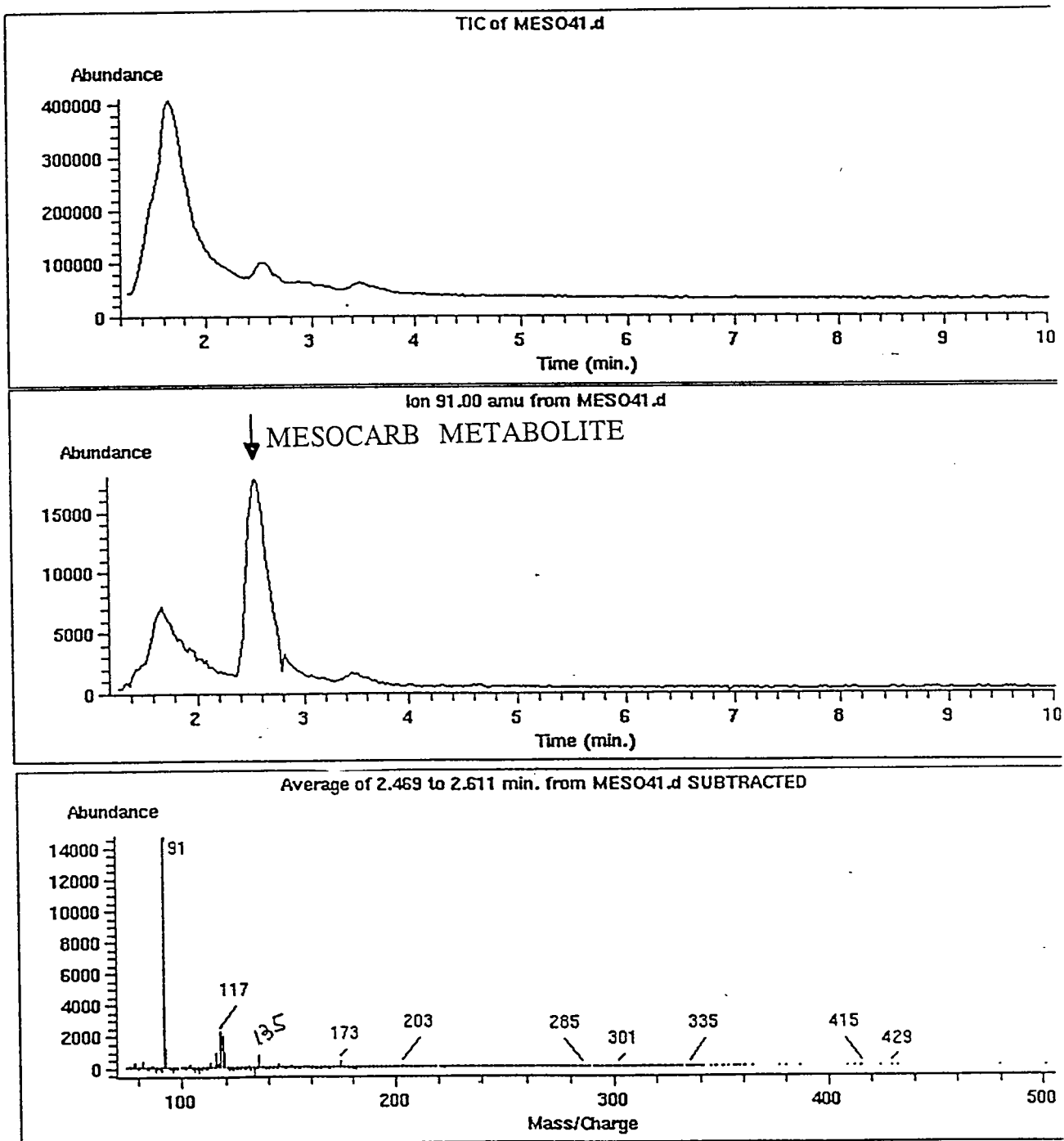


Figure 11. Analysis by LC/MS of a non hydrolysed urine extract from an excretion study of mesocarb. TIC, chromatogram of m/z 91 and mass spectrum of the peak detected.

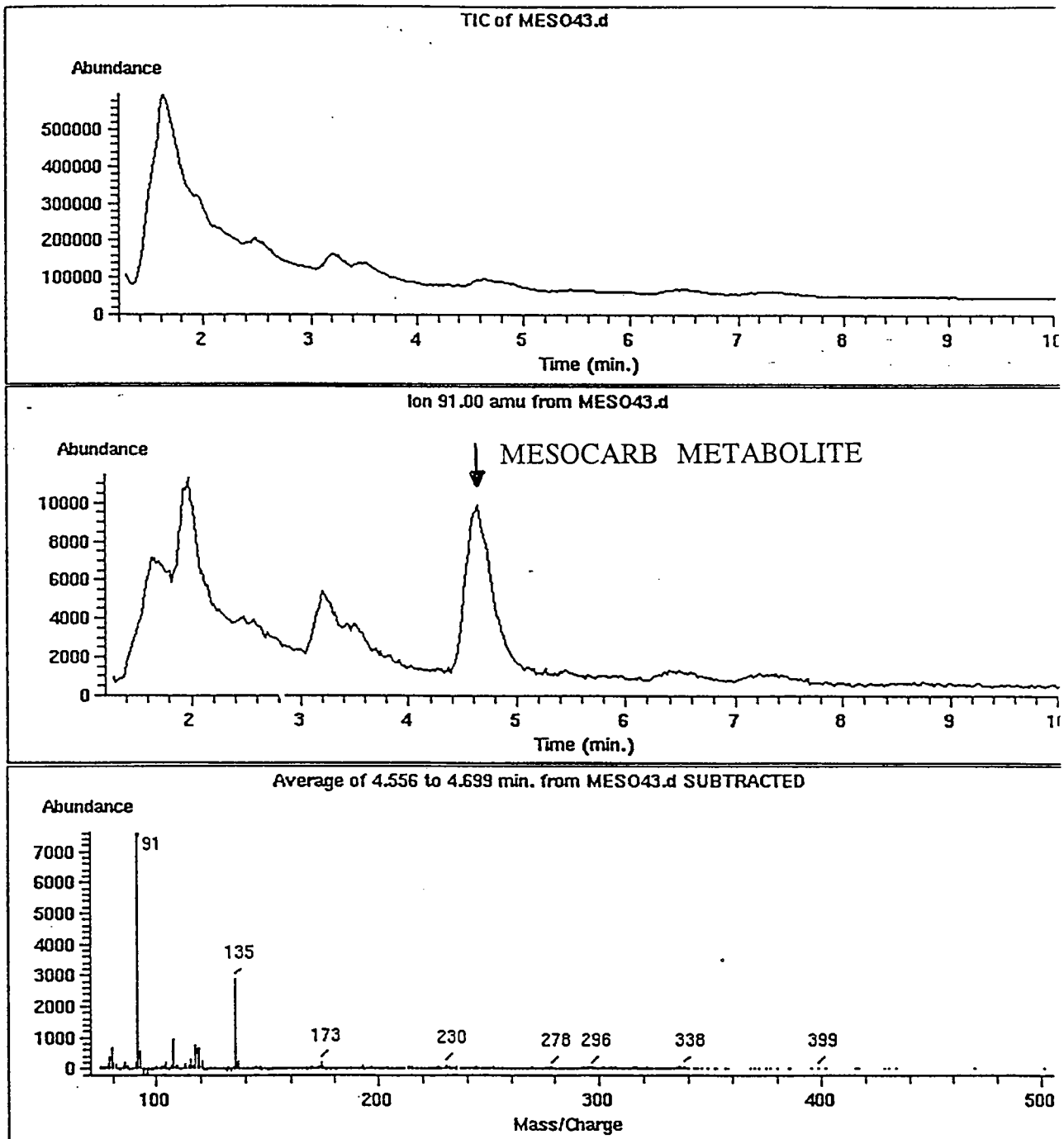


Figure 12. Analysis by LC/MS of an hydrolysed urine extract from an excretion study of mesocarb. TIC, chromatogram of m/z 91 and mass spectrum of the peak detected corresponding to p-hydroxy-mesocarb.